

CALCIUM OXALATE HYDRATES IN *DRACAENA SANDERIANA* HORT. SANDER
EX M.T. MAST. (DRACAENACEAE) AND THEIR RELEVANCE TO THE FIELD OF
BIOMINERALIZATION

By

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by

Svoboda V. Pennisi

This work is dedicated to my stepfather, who introduced me to the incredible
world of science

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Dracaena sanderiana hort Sander ex M.T. Mast. possessed a variety of biogenic calcium oxalate deposits in both intracellular compartments and extraplasmic chambers. Formation of calcium oxalate crystals in leaves of *Dracaena sanderiana* was highly specific and predictable with respect to the location of the various crystal types and relative timing of their development during leaf ontogeny. The hydration state of cuticular crystals was calcium oxalate monohydrate (COM). The observed polarity in some crystals with respect to their cuticular orientation, the existence of crystal envelopes, and preferential orientation of the crystallographic axes strongly suggested biologically controlled crystal precipitation. The crystal origin was *extraplasmic* since the epidermal cell wall is external to the COM crystals. This finding suggested that cuticular crystallization of COM in *D. sanderiana* occurred in crystal chambers which originated from the plasma membrane. Vesicles

derived from the rough endoplasmic reticulum (RER) were actively involved in crystal deposition process.

Intracellular calcium oxalate dihydrate (COD) crystals in *D. sanderiana* displayed typical tetragonal-dipyramidal morphology although development of some unusual crystal faces occurred. Precipitation of COD inside *D. sanderiana* cells was highly controlled as evidenced by the elaboration of RER crystal vacuoles and the fact that crystal morphology was modified by the development of unstable crystal faces. Raphide-containing cells in *D. sanderiana* exhibited characteristics typical of System II crystal idioblasts with lamellate sheaths around the chamber walls, mucilage-like material surrounding the developing crystal chambers, and paracrystalline bodies with closely-spaced subunits.

Dracaena sanderiana plants responded to exogenous Ca^{2+} levels in remarkably different ways depending on whether they had been deprived of endogenous Ca^{2+} . Calcium-deficient plants deposited cuticular crystals at the expense of the intracellular raphide bundles. The total number of extraplasmic crystals per epidermal cell in calcium-deficient plants grown in nutrient solutions with and without calcium were similar, but crystal size was considerably smaller when the nutrient solutions did not contain calcium. This finding implied that nucleation sites were pre-determined and finite in number. In contrast, intracellular raphide idioblasts were highly variable in induction and numbers as compared to the extraplasmic crystals.

The present study involved 14 species of *Dracaena*. Detection of cuticular crystals in all species examined indicated that these crystals were probably ubiquitous in the genus. Species of the 'Tree Dracaenas' deposited the largest quantity of uniformly small cuticular crystals. The distinction among

species within this group, based solely on crystal numbers and size, was not reliable. Other species of *Dracaena* studied displayed variable quantity and size of cuticular crystals. This fact, together with leaf epidermal characteristics, could be taxonomically valuable in the genus *Dracaena*.

CHAPTER 1 INTRODUCTION

Crystalline deposits are a common feature throughout the plant kingdom. Phytocrystals are composed of a variety of chemical compounds with the most common compounds being calcium oxalate (CO) hydrates, calcium oxalate monohydrate (COM), and calcium oxalate dihydrate (COD). Typically crystals of CO appear intracellularly in specialized cells called crystal idioblasts. Two general systems based on the presence or absence of membranes and associated subcellular structures are recognized in angiosperm species (Horner and Wagner, 1995). System I is typical of dicotyledonous species, and involves cytoplasmic spherosomes, vacuolar organic paracrystalline bodies with widely spaced subunits, membrane complexes, plasmalemmasomes, and crystal chambers. System II is characteristic of monocotyledonous species; although it lacks vacuolar membrane complexes and the paracrystalline bodies display closely spaced subunits.

Extracellular crystal deposition involves the deposition of a crystalline substance outside of the plasma membrane, although such deposition should be termed extraplasmic because the cell wall is part of the cell. In some cases the crystals are exterior to the cell wall, although in others, they are partially or fully embedded in the cell wall. In this study all citations pertaining to crystals

exterior to the cell wall apply terminology used by the authors. In all other instances crystals are reported as "extracellular".

Extracellular calcium oxalate crystal deposition is a characteristic feature of numerous gymnosperm species. Oladele (1982) used the term "crystalliferous cuticle" to describe the deposition of small calcium oxalate crystals embedded in the epidermal cell wall of *Chamaecyparis lawsoniana*. The ontogeny of the extracellular deposits in coniferous gymnosperms indicates extracellular origin (Oladele, 1982; Fink, 1991a).

Reports of extracellular crystal formation in angiosperms are scarce and only a few are detailed. *Nymphaea* is a classical example of an angiosperm genus in which specialized astrosclereid cells exhibit extracellular calcium oxalate crystals located between the primary and secondary cell walls (Arnott and Pautard, 1970). The crystallization process commenced within crystal chambers that are situated between the plasma membrane and the primary cell wall (Kuo-Huang, 1992). The primary site of initial crystallization of extracellular crystals in *Dracaena marginata* and *Sempervivum wulfenii* is cytoplasmic with later translocation to the cell walls (Fink, 1991b). The mode of crystal deposition with respect to the cell wall and the cytoplasm in angiosperms differs dramatically to that which occurs in coniferous gymnosperms. In addition, subcellular structures such as crystal chambers, do not always occur in the latter plant group.

Dracaena sanderiana hort Sander ex M.T. Mast. possesses a variety of biogenic calcium oxalate deposits in both intracellular compartments and the extracellular apoplastic space (Vladimirova, 1996). Presently it is unknown whether this plant shares developmental features similar to other angiosperm

species with respect to its intracellular and extracellular crystalline deposits. The objectives of this study are: (1) to characterize the chemical nature and crystallographic properties of the calcium oxalate hydrate forms in *Dracaena sanderiana*; (2) investigate the developmental aspects of extracellular epidermal crystal deposition; (3) study the effects of exogenous calcium supply on crystal deposition and the partitioning of calcium oxalate among the different crystal types; (4) determine the value of extracellular epidermal crystals to separate taxonomically species within the genus *Dracaena*.

CHAPTER 2 LITERATURE REVIEW

Biom mineralization

Living organisms produced minerals via a variety of processes collectively termed biom mineralization (Lowenstam and Weiner, 1989). This biological phenomenon was fundamentally important, given its widespread occurrence and impact on the biosphere. All five kingdoms (i.e., the Monera, Protista, Fungi, Plantae, and Animalia), had representatives which formed biogenic minerals (Lowenstam and Weiner, 1989). The organisms which evolved the capability to precipitate minerals gained an evolutionary advantage due to the superior mechanical strength of protective shells imparted by the biom mineralization products (Lowenstam and Weiner, 1989).

Two processes were involved in the formation of biogenic minerals, namely: (1) "biologically induced" precipitation and (2) "biologically controlled" precipitation (Mann, 1983). In the first process minerals formed as a result of interactions between the activity of the organism and its proximal environment. In this process the control exerted by the organism over the biom mineralization process was minimal and typified by bulk extracellular and/or intracellular precipitation without the presence of organic matrices. In contrast, "biologically controlled" precipitation implied implementation of organic matrices which were under rigid genetic control. Characteristic of this biom mineralization process was an array of cellular activities directed towards structural, spatial,

and chemical control over mineral deposition. Both the extracellular matrix and intracellular compartments could serve as a site for mineral precipitation.

Intravesicular deposits could be transferred to an extracellular location or they could serve as accumulation centers for aqueous ions later transported to an extracellular mineralizing site (Mann, 1983).

Recognition of "biologically induced" versus "biologically controlled" types of mineral deposition was crucial to studying any organism involved in the biomineralization process. The latter process was infinitely more intriguing because it actively involved the living system and offered a challenge to our understanding of how organisms selected, localized, and concentrated elements. The process also might help us understand how minerals were nucleated, spatially segregated, their internal microstructure and bulk shape determined, and how inorganic/organic interfaces were controlled (Birchall, 1989).

The occurrence of biogenic minerals in Kingdom Plantae has been extensively recorded. The following was a list of mineral types and the corresponding mineral names shown to occur in plants: crystalline calcium carbonates (calcite, aragonite, vaterite) and amorphous calcium carbonates, silica, iron oxide (ferrihydrate), citrate (earlandite), calcium oxalates (whewellite, weddellite), calcium tartrate and calcium malate (Lowenstam and Weiner, 1989). The most common mineral types in plants were the CO₂ hydrates, whewellite and weddellite.

Calcium Oxalate in Higher Plants

Calcium and Oxalic Acid in Higher Plants

Calcium moved by mass flow through the xylem, although active absorption has also been reported (Franceschi and Horner, 1980a). According to Fink (1991a), calcium (Ca^{2+}) had a dual role in plant nutrition. It was an integral part of the cell wall (Ca-pectate) and membranes (Ca^{2+} ions bridge phosphate and carboxylate groups of phospholipids and proteins). It also was an intracellular second messenger in signal transduction. However, in higher concentrations, calcium interfered with the energy metabolism as it binds to inorganic phosphate. Hence, cytoplasmic concentrations of Ca^{2+} were kept at physiologically safe levels, and free cytosolic calcium levels were low ($< 1\mu\text{M}$). These levels were maintained by the removal of Ca^{2+} either by the restriction of cytosolic entrance or the sequestration in intracellular compartments (vacuole, endoplasmic reticulum) (Bangerth, 1979; Trewavas, 1986; Fink, 1991a).

Oxalic acid balanced Ca^{2+} and other ions in plants (Franceschi and Horner, 1980a). A close relationship between oxalic acid formation and Ca^{2+} uptake existed. Fink (1991a) hypothesized that oxalate anions serve to precipitate excess Ca^{2+} and render it insoluble. This precipitation occurred intracellularly (inside vacuoles) and extracellularly (outside the plasmalemma), and led to the formation of crystalline deposits. Oxalic acid has been a subject of detailed research and has been considered an end product of metabolism (Franceschi and Horner, 1980a). Its synthesis in plants has been linked to photosynthesis, as either a direct or an indirect product. Oxalic acid concentration increased during illumination of the plant and decreased during

dark periods (Pucher et al., 1939). Others have since suggested that oxalate synthesis was related to photosynthetically-derived carbon compounds, and was formed from precursors synthesized in the light (Stutz and Burris, 1951). One of the first products of photosynthesis, glycolic acid, was converted to oxalic acid with glyoxalic acid as an intermediate (Nord and Vitucchi, 1947).

The major pathways for oxalate synthesis were summarized in Figure 2-1. Seal and Sen (1970) proposed a relationship between photosynthesis and oxalic acid production. Photosynthetically-derived glycolate was transported into the peroxisome and was oxidized by glycolate oxidase to glyoxylate, which was further oxidized to oxalic acid by the same enzyme or via xanthine oxidase (Figure 2-1). Glycolic acid oxidase was found in 16 common plant families (Noll and Burris, 1954). Glycolate and glyoxylate were the immediate precursors of oxalic acid in some plants (Franceschi and Horner, 1980a). The oxalate was channelled into the vacuole and can be stored in soluble or an insoluble form (i.e., CO deposits). Carbon compounds not directly related to photosynthesis also produced oxalic acid in some plants. In *Oxalis* sucrose could be a precursor for the oxalate (Millerd et al., 1963), and in *Begonia* oxalate could be derived from glucose (Tavant, 1967). Oxalate also could be synthesized from stored carbohydrates via the Krebs cycle in the mitochondria (Figure 2-1). Kornberg and Krebs (1957) demonstrated that isocitric acid, via enzymatic cleavage involving isocitrate lyase, could give rise to glyoxalate and succinic acid, with the former involved in oxalate synthesis. L-ascorbic acid, which can be synthesized from D-glucose, was also involved in oxalate synthesis in some plants (Wagner and Loewus, 1975).

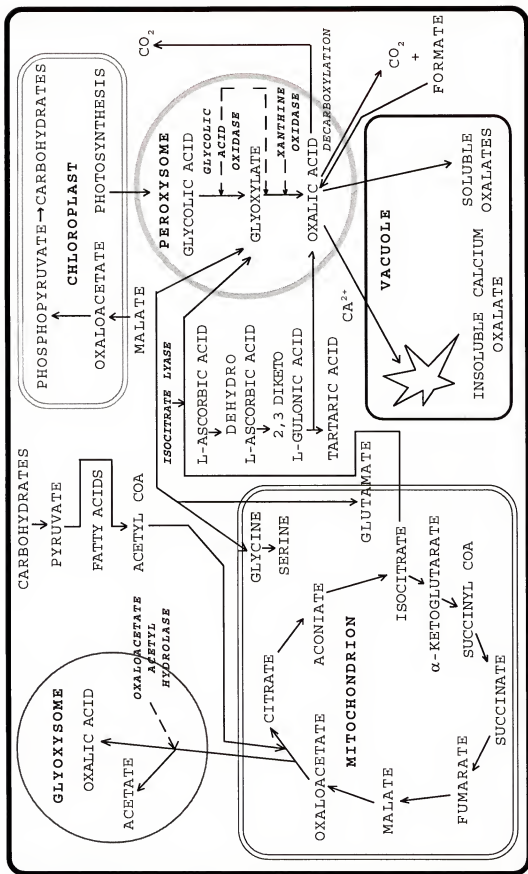


Figure 2-1. Summary of metabolic pathways of oxalic acid production in plants (redrawn from Franceschi and Horner, 1980a).

Oxalic acid also could arise from direct oxidation of malic acid (Gentile, 1954) and by enzymatic cleavage of oxaloacetate in the Krebs cycle (Hayaishi et al., 1956).

Calcium Oxalate Crystal Terminology, Location, and Taxonomic Significance

Terminology

Haberlandt (1914) stated the five commonly found calcium oxalate (CO) crystals in plants:

1. raphide – a needle-shaped crystal encountered in bundles
2. styloid – an elongated crystal with pointed or ridged ends
3. prisms – variously shaped
4. crystal sand – a mass of numerous very small individual crystals
5. druse – spherical aggregate of variously-shaped individual crystals.

Chattaway (1955, 1956) listed somewhat different descriptions of some of these five crystal categories and added another type in woody tissues:

1. druses – spherical aggregate of variously-shaped individual crystals
2. raphides – a needle-shaped crystal encountered in bundles
3. styloids – "elongated and rod-like" – "elongated crystals about four times as long as broad, with pointed or square ends; rod-like similar to the preceding in general shape, but only about twice as long as broad, and usually with square ends"
4. "acicular" – "needle-shaped crystals, often rather small, not bundled, but lying free in the cells and not filling them"
5. crystal sand – a mass of numerous very small individual crystals

6. "rhomboidal, square or diamond-shaped."

Clearly, crystal descriptions from different researchers could vary considerably. Various other descriptions of crystal shape existed such as "tri-radiate," "irregular" (Buss and Lersten, 1972), "pyramidal," "rhomboid," "flat rhomboidal," "lenslike" (Okoli and Green, 1987), and "spherulite" (Horner and Wagner, 1992). Terminology should be compared with illustrations because occasional misunderstandings could occur. For example, Gornall (1987) described "needle-like" crystal in *Saxifraga nipponica*, when a SEM image revealed large, acicular morphology more appropriately categorized as a styloid. Dahlgreen and Clifford (1982) considered styloids as pseudoraphides (i.e., elongated prismatic crystals that might attain an almost needle-like form). Somewhat poetic description of phytocrystals was given by Calmes and Carles (1970), who described CO in *Parthenocissus tricuspidata* as resembling "tiny sea urchins."

Crystal Location Within the Plant Body

Calcium oxalate deposits could be found in virtually every organ, tissue, and cell type of the plant body (Francheshi and Horner, 1980a). Some examples for unusual tissue locations were the vascular cambium (Rao and Dave, 1984), bark (Trockenbrodt, 1995), and sieve cells of the secondary phloem and phelloderm (Chau, 1986). Horner and Wagner (1995) recognized the following general crystal locations with regard to the cytoplasm and the cell wall: the cell vacuole (most frequent), storage organelles, as seed storage bodies (Buttrose and Lott, 1978), and less commonly, starch grains (the crystals were termed

intra-amylar) (Okoli and Green, 1987), the cell wall, and external to the cell wall.

Taxonomic Significance

Calcium oxalate crystals occurred in more than 200 families of angiosperms (Zindler-Frank, 1976) and gymnosperms (Fink, 1991a). Some researchers attached limited taxonomic value to crystals for certain plant species due to their identifiable shape(s) and location(s) within the plant body (Horner and Wagner, 1995). Buss and Lersten (1972), however, could not find any consistent patterns that conformed to presently defined taxa when they surveyed tapetal crystals in 84 species of 52 genera of Leguminosae.

The taxonomic importance of CO crystals has also been questioned by Scurfield and Michell (1973), who stated that only taxonomic generalizations of a very broad type can be made, e.g., woody monocots tended to form raphides. Their arguments were that a single plant could form several types of crystals, sometimes in adjacent cells, and crystal habit could be altered easily by physical conditions. According to Scurfield and Mitchell (1973), impurities contained within the cell might have affected crystal shape because different types of cells might have different chemical composition that result in variously shaped crystals.

Intracellular Deposition of Calcium Oxalate in Higher Plants

As mentioned earlier, CO crystals occurred most frequently in the cell vacuole. Usually conspicuously larger than the surrounding cells, the crystal-containing cell was termed a *crystal idioblast*. Crystal deposition within

vacuoles was characteristic of angiosperms. Horner and Wagner (1995) proposed two general systems based on the presence or absence of membranes and associated subcellular structures. System I (Figure 2-2) was exemplified by druses in *Capsicum* and *Vitis*, raphides in *Psychotria* and crystal sand in *Beta*. System I crystal idioblast formation involved cytoplasmic spherosomes, vacuolar organic paracrystalline bodies, membrane complexes, plasmalemmasomes, and crystal chambers. The vacuolar paracrystalline bodies exhibited subunits with large periodicity and were linked to a membrane network which formed the crystal chambers. Small tubules (10-13nm) around the developing raphide chambers were presumed to assist in orientation and alignment of the elongating crystal chambers. Corresponding tubules of this type were not found in *Capsicum* druses or in System II idioblast types. Notably the described features of System I were observed only in dicotyledonous species.

System II (Figure 2-3) was exemplified by the monocotyledonous raphide idioblasts in *Typha*, *Vanilla*, and *Yucca*. System II lacked vacuolar membrane complexes and the paracrystalline bodies displayed closely spaced subunits. Crystal chamber walls were believed to form as interface aggregations of flocculent material. Mucilage-like material was present around the developing crystal chambers and lamellate sheaths were observed around the chamber walls (Wattendorff, 1976; Horner and Wagner, 1995).

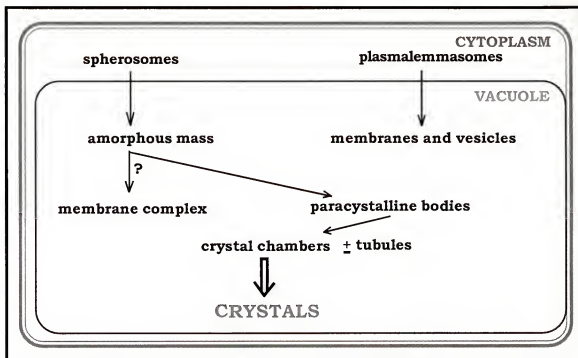


Figure 2-2. Schematic representation of System I crystal idioblast (redrawn from Horner and Wagner, 1995).

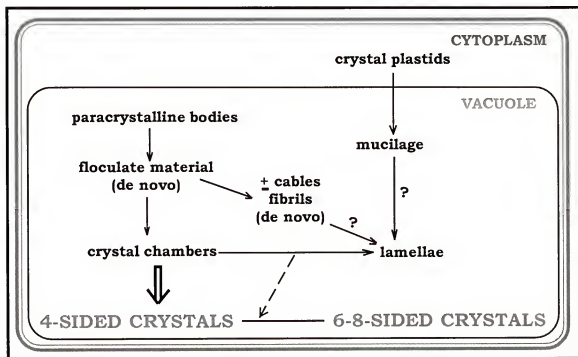


Figure 2-3. Schematic representation of System II crystal idioblast (redrawn from Horner and Wagner, 1995).

Extracellular Deposition of Calcium Oxalate in Higher Plants

Coniferous Gymnosperm Species

Extracellular CO crystals, in various degrees of attachment to the cell wall, were a feature commonly found in coniferous gymnosperms. Fink (1991a) reported extracellular deposition of CO in various tissues in acicular leaves of species of the Araucariaceae, Cupressaceae, Podocarpaceae, Pinaceae, Taxaceae, and Taxodiaceae. Crystals occurred on the outer surfaces of palisade and spongy cell walls in hemlock (Gambles and Dengler, 1973). More recently, Carlquist and Gowans (1995) reported minute CO crystals lining the intracellular spaces in the vascular rays of *Ephedra*.

Frey-Wyssling (1935) doubted extracellular crystallization of CO occurred because a plant cell would have to excrete Ca^{2+} and oxalic acid through different pathways into the apoplast, where crystallization would then have to occur immediately upon contact. Conversely, Fink (1991a) argued that if Ca^{2+} ions moved mainly with the mass flow of water within the wall and bound reversibly to exchange sites in the cell wall, the cell only needed to secrete oxalic acid externally to precipitate CO in the apoplastic space.

The primary site of crystallization varied even within tissues of the same plant. Minute crystals originated in the radial walls of pine needle phloem. As tissue aged, tearing forces caused separation of the radial walls thus forming large intracellular spaces, where crystals were subsequently deposited (Fink, 1991b). According to the same author, most crystal growth in the mesophyll began in the outermost layer of the cell wall. Upon examination of crystal

development in several coniferous gymnosperms, Fink concluded that the crystals originated *in situ* within the cell wall.

Oladele (1982) described the formation of crystalliferous cuticle in *Chamaecyparis lawsoniana*. The development of extracellular crystals was associated with a "membranous crystal-initiating structure" (Figure 2-4). Periclinal growth of small peripheral crystals within the membranous compartment, and subsequent coalescing formed a single crystal approximately 0.7 μm high and approximately 2 μm long (as seen in transverse section of an epidermal cell). The final shape of the crystal was typically parallelogram with rounded corners. The crystal formation might be linked to wall evaporation, and the membranous crystal-initiating structures might be "precipitation membranes." Cuticular transpiration might have created a zone of high concentration of Ca^{2+} ions in the cell wall, while oxalic acid in the cytoplasm might have caused high concentration of oxalate ions near the plasmalemma (Oladele, 1982). The two poles of oppositely charged ions within the outer periclinal wall and their counter-diffusion might have created spontaneous formation of "precipitation membranes."

Angiosperm Species

Compared to gymnosperms, extracellular CO crystals did not commonly occur in angiosperms. Reports of extracellular deposition in angiosperms were scarce and often disputed. Franceschi and Horner (1980a) speculated that CO crystals were originally formed intracellularly in the cytoplasm and later displaced in the extracellular space. The CO crystals in the epidermis of *Stylosanthes guianensis* were a well-known example of the extracellular

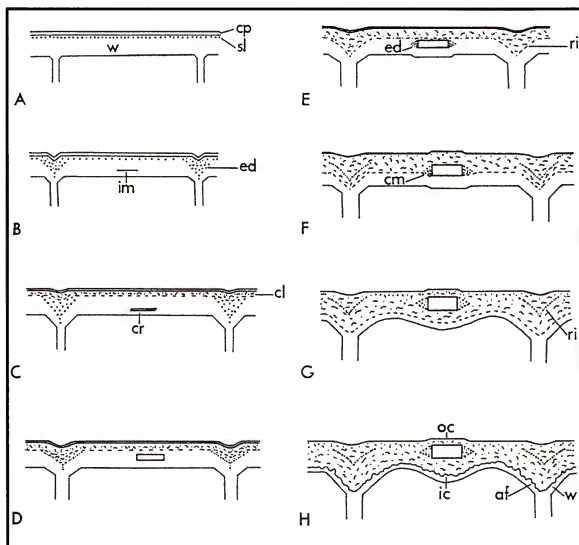


Figure 2-4. Schematic representation of the developmental stages in the formation of crystalliferous cuticle in *Chamaecyparis lawsoniana* (redrawn from Oladele, 1982).

Figure abbreviations: af, anticlinal flange; c, cuticle; cl, cuticular layer; cm, crystal cuticular ramp; cp, cuticle proper; cr, crystal; ed, electron-dense deposit; ic, inner crystal tubercle; im, crystal initiating membrane; oc, outer crystal tubercle; ri, cutinized ridge; w, wall.

appearance of crystals formed intracellularly in an angiosperm (Brubaker and Horner, 1988). The endogenously formed twin prismatic crystals in this species became embedded in cell debris upon the demise of the epidermal tissue surrounding them.

Berg (1994) conclusively demonstrated CO crystals embedded in the outer cell wall that comprised the branchlet surfaces of Casaurinaceae species. The appearance of these deposits soon after the epidermis was formed suggested that the mechanism for crystal induction might not be identical to the one for intracellular crystal idioblasts.

Borchert (1984) reported the existence of large numbers of very small, irregular crystals in the epidermal cell walls in honey locust (*Gleditsia triacanthos*). Okoli and McEuen (1986) found small crystals on the walls of the vessel members in *Telfairia pedata* and *T. occidentalis* (Cucurbitaceae). The angiosperm *Nymphaea tetragona* was another noteworthy example, where extracellular crystals occurred embedded between the primary and secondary wall of specialized cells called astroclereids (Kuo-Huang, 1992; Arnott and Pautard, 1970). Crystallization commenced within crystal chambers that were presumably extensions of the plasma membrane. These chambers were situated between the plasma membrane and the primary cell wall. After secondary cell wall material was deposited, the crystals became embedded between the primary and secondary cell walls (Kuo-Huang, 1992).

The primary site of crystallization of extracellular crystals in the angiosperms *Dracaena marginata* and *Sempervivum wulfenii* was from "deeper layers within the cell walls" in the former, whereas crystals were initially seen as "free-floating in the cytoplasm," and then secondarily attached to the

internal cell walls, and covered by additional wall material in the latter species (Fink, 1991a).

The presence of crystals between the epidermal cell wall and the cuticle was considered a typical adaptive feature for xerophytic habitats (Figure 2-5) (Ihlendfelt and Hartmann, 1980).

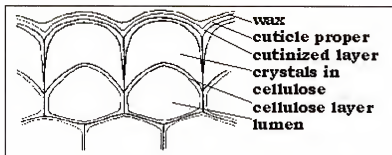


Figure 2-5. Typical features of a xeromorphic type of epidermis (redrawn from Ihlendfelt and Hartmann, 1980).

Crystalline incrustations occurred in the outer epidermal wall of several species of Mesembryanthemaceae (Figure 2-6). These deposits were classified in three categories according to size: crystal sand (less than and up to $1\mu\text{m}$ in diameter), crystals ($2 - 5\mu\text{m}$ in diameter), and crystal druses (larger than $5\mu\text{m}$ in diameter). The last category implied that single crystals could not exceed $5\mu\text{m}$ in diameter, however, druses have been defined as crystal aggregates. The crystal deposits were shown embedded in the cellulosic layer in the outer tangential walls of the epidermal cells (Figure 2-6).

Transmission electron micrographs of developing epidermal cells in *Casaurina* showed large amounts of endoplasmic reticulum (ER) associated with the plasmalemma (Berg, 1994). A positive relationship existed between the amount of ER and the stage of secretion of CO.

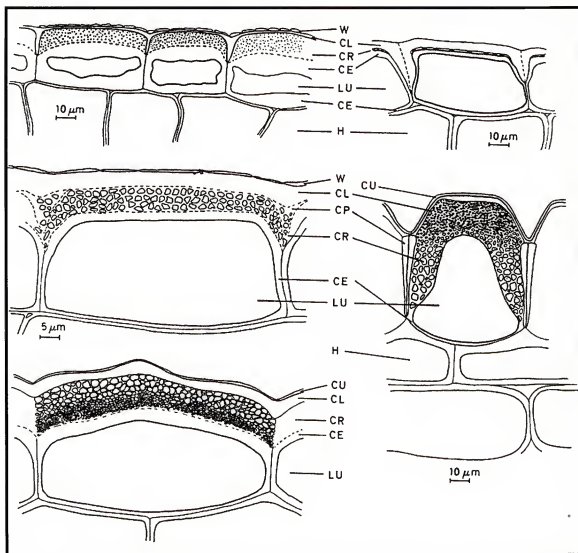


Figure 2-6. Calcium oxalate incrustations in the outer epidermal wall of Mesembryanthemaceae species. Drawings represent transverse views of epidermal cells.

Figure bbreviations: w, wax; cl, cutinized layer; cr, crystal layer; ce, cellulose layer; cp, cutinized peg; cu, cuticle proper; h, hypodermis; lu, lumen (redrawn from Ihlenfeldt and Hartmann, 1980).

Cells in which the secretion process was completed contained considerably less ER compared to cells in which the process was ongoing.

Chemical Composition and Crystallography of Calcium Oxalate Hydrate in Higher Plants

General Characteristics of Calcium Oxalate Hydrate Forms

When Ca^{2+} and oxalate ions were brought together in a neutral environment, the precipitate was a mixture of three hydrates – monohydrate ($\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$) (COM), dihydrate ($\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) (COD), and trihydrate ($\text{CaC}_2\text{O}_4 \cdot 3\text{H}_2\text{O}$) (COT). COM and COD existed in nature as the minerals whewellite and weddelite, respectively. The monoclinic COM was the least soluble and thermodynamically most stable phase, the triclinic COT was the most soluble phase and undergoes transformation to COM (Tomažic and Nancollas, 1980). The tetragonal COD also transformed to COM. The triclinic system had three crystallographic axes (a , b , and c) of unequal length that all intersected at oblique angles (Figure 2-7A) (Klein and Hurlbut, 1993). The angles between the axes, by convention, were α , β , and γ (Figure 2-7). The monoclinic system had three unequal crystallographic axes, two of which were inclined to each other at an oblique angle and the third perpendicular to the plane of the other two (Figure 2-7B). The tetragonal system had three mutually perpendicular axes, two of which (the horizontal axes) were of equal length (a_1 and a_2), while the vertical axis was shorter (Figure 2-7C1) or longer than the other two axes (Figure 2-7C2).

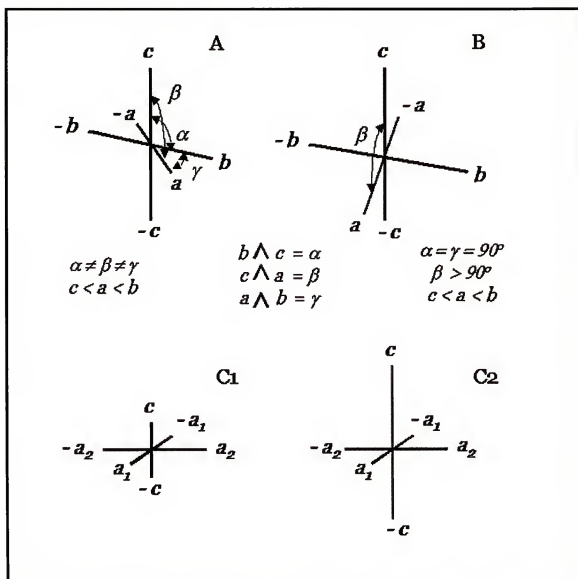


Figure 2-7. Crystallographic axes, angles and their relationships in triclinic (A), monoclinic (B), and tetragonal (C) crystal systems (adopted from Klein and Hurlbut, 1993).

The crystallographic axes were imaginary reference lines that generally were taken parallel to the intersection edges of major crystal faces. Such axes primarily were determined by symmetry and coincided with symmetry axes or normals to symmetry planes. The internal symmetry increased from the triclinic system (lowest degree of symmetry) and the monoclinic (intermediate degree of symmetry) to the tetragonal (highest degree of symmetry). The triclinic system had only a 1-fold rotoinversion axis; the monoclinic system had in addition to a 1-fold rotoinversion axis, one 2-fold rotation axis and one mirror plane, and the tetragonal system had a 1-fold rotoinversion axis, one 4-fold rotation axis, five 2-fold rotation axes, and five mirror planes. Based on crystal morphology, the triclinic and monoclinic systems could be easily distinguished from the tetragonal system. The separation of the triclinic from the monoclinic system on the basis of crystal morphology, however, was difficult.

Crystal faces were defined by indicating their intercepts on the crystallographic axes (Klein and Hurlbut, 1993). Thus, in describing a crystal face it was necessary to establish whether it was parallel to two axes and intersected the third, was parallel to one and intersected the other two, or intersected all three. Furthermore, their relative distance to the face that intersected the three axes must be determined. Finally, after all face intercepts have been established, a set of whole numbers describing a crystal face was derived (i.e., the Miller indices (Figure 2-8)).

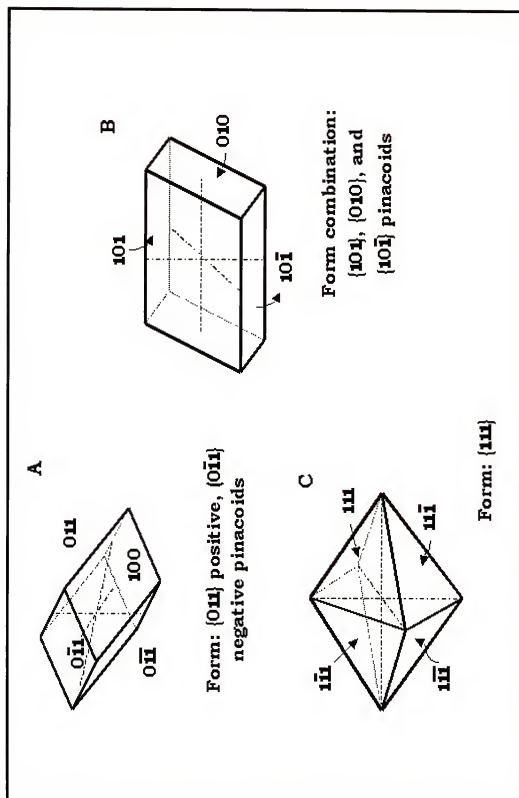


Figure 2-8. Examples of three crystal forms and Miller indices for the crystal faces of triclinic (A), monoclinic (B), and tetragonal (C) crystal systems (adopted from Klein and Hurlbut, 1993).

Characteristics of Calcium Oxalate Hydrate Forms in Higher Plants

Frey-Wyssling (1981) considered that the two principal forms of CO crystals occurring in plants were monohydrate and polyhydrate. Plants actually precipitated "tetragonal polyhydrate" with the formula $(\text{CaC}_2\text{O}_4 \cdot (2+x)\text{H}_2\text{O})$. He argued that the "trihydrate" from other literature sources was a dihydrate complemented by zeolitic water, which could leave the crystal lattice upon drying so that analyses produced variable results (Philipsborn, 1952). It was generally agreed that the COM form was the most common hydrate of CO found in plants. Information on COT in plants was virtually nonexistent, conceivably due to the transient nature of this phase which precluded isolation and crystal determination. Reliable tests that could distinguish between the hydrate forms were X-ray diffraction analysis, electron diffraction, and to a certain degree, polarizing optics.

Polarizing optics relied on the birefringence of a crystalline substance that was an intrinsic property with a high diagnostic value. Plant CO crystals had widely varying dimensions, and the birefringence analysis required prior knowledge of crystal thickness which was difficult to assess strictly from light microscopy images. Many extracellular crystals were minute and standard crystallographical analysis of birefringence was difficult. Under cross-polarized light the polyhydrate phytocrystals showed interference colors of only gray to white of the first order (Frey-Wyssling, 1981). In contrast, the monohydrate phytocrystals displayed multicolored interference of the second and third order. The birefringence of a crystalline substance increases with thickness, so that even the low birefringence dihydrate (polyhydrate) can display colors of second and third order in some instances (Pennisi, personal comm.).

Relationship Between Crystal Morphology and Hydration State of Calcium Oxalate in Higher Plants

In their review article Franceschi and Horner (1980a) provided an exhaustive list of crystal morphologies from various plants along with the hydration state and the method used for determination. Raphide morphology was typically the COM form, while the druse and prismatic morphologies could be either COM or COD. Styloids were consistent with the COM form. Reports of "solitary" vs. "prismatic" crystal type were confusing since prismatic crystals were often solitary. By the same logic "conglomerate" crystal type could not be readily separated from "druse" type because the latter was a compound crystal. Calmes and Carles (1970) gave a conflicting account of two types of CO crystals found in Boston Ivy (*Parthenocissus tricuspidata*), "White needles gathered in raphides" were the trihydrate form (the "tetragonal" hydration form of CO), and "tiny sea urchin"-shaped crystals of the monohydrate form. The latter type probably was a COM druse. Since no X-ray data was provided, this single account of trihydrate raphides remained questionable. Conclusive identification of CO hydrate form(s) along with precise measures of crystal dimensions perhaps was a better way of describing the type of crystal(s) that occurred in the plant under investigation.

Conditions for the Formation of COM vs. COD

At temperatures around 15 °C polyhydrate CO was formed in cells when the cell sap had high concentrations of Ca^{2+} , and oxalate ions were introduced. COM forms where cells predominantly transported Ca^{2+} ions into an oxalate-rich environment (Frey-Wyssling, 1935). In an extensive survey of 390 plant

families known to possess CO crystals, McNair (1932) concluded that the monohydrate form prevails in tropical families, while the trihydrate (polyhydrate) form was equally distributed among tropical and temperate families. According to Scurfield and Michell (1973), the hydration form and crystal morphology could be attributed to chemical composition, which differed from cell to cell.

Crystal Deposition as Affected by Calcium Supply

The amount of crystals formed in a plant could be altered by various factors. For example, high humidity and high Ca^{2+} fertilizer increased the formation of CO crystals in tomato fruits (De Kreij, 1992). In the same study an increased phosphate supply increased crystal quantity through increased Ca^{2+} uptake. Experiments with *Canavalia ensiformis* (Frank, 1972) showed that the number of crystal idioblasts decreased by 50% when plants were grown with low calcium ($0.2 \text{ meq/Ca}^{2+}/\text{l}$). Although plants showed signs of Ca^{2+} deficiency at this concentration, crystal idioblasts still formed. Franceschi (1989) reported that CO formation in root raphide bundles of *Lemna minor* was rapidly induced (artificially raised exogenous Ca^{2+}) and reversible (using Ca^{2+} -ionophore).

Xylem sap of spruce trees grown on calcareous soil (18 times more Ca^{2+} compared to Ca^{2+} -poor soil) contained only twice the Ca^{2+} concentration of xylem sap from spruce trees grown in Ca^{2+} -poor soil (Kartusch et al., 1991). However, spruce trees grown on Ca^{2+} -rich soil had considerably more crystals than trees grown on a Ca^{2+} -poor soil.

No known studies have explored the effects of Ca^{2+} supply in a plant system featuring two or more CO types in several locations of the plant body.

Such a system might provide insights into Ca^{2+} partitioning among the crystal types and information on the relative priority and relationships among the various CO hydrates.

Functional Aspects of Calcium Oxalate in Higher Plants

General Functions

Franceschi and Horner (1980a) suggested that CO crystal formation might serve as a means of ionic detoxification. Fink (1991a) argued that the Ca^{2+} had to be sequestered to maintain low cytosolic concentrations. Frey-Wyssling (1981) supported the view that the "poisonous" oxalate had to be removed from the cytoplasm. Franceschi and Horner (1980a) presented a more complete hypothesis which linked oxalic acid synthesis, nitrogen metabolism, ionic balance, and Ca^{2+} absorption. Plants that utilized nitrate nitrogen produced net OH^- during nitrate assimilation, which tended to raise pH of the cell sap. Oxalic acid could neutralize OH^- ; however, an increased production of oxalic acid would disturb the ionic balance and result in an increased osmotic pressure. The excess acid was effectively removed by precipitation as a Ca^{2+} salt. Oxalic acid was also formed when an imbalance of ionic charges occurred, e.g., during growth on high Ca^{2+} medium.

Calcium oxalate crystals could serve as means for protection against herbivory by imposing considerable constraints for phytophagous insects (Kimmerer and Potter, 1987). Leafminers avoid crystal-containing cells in leaves of *Ilex opaca* (Kimmerer and Potter, 1987). Calcium oxalate crystals were shown to act as Ca^{2+} storage facilities (Webb and Arnott, 1982) and also provided structural strength (Tilton and Horner, 1980). Calcium oxalate druses

in *Lycopersicon* might assist in the weakening and dissolution of the septal cells and anther dehiscence (Bonner and Dickinson, 1989). Kausch and Horner (1981) suggested that CO crystal idioblasts in *Typha angustifolia* played a role in the schizolysigenous formation of air spaces by regulating Ca^{2+} ion flux in the vicinity of the aerenchyma cells. Horner and Wagner (1995) mentioned a putative function of the crystals in the gathering and reflection of light.

Function of the Extracellular Crystal Deposits

In contrast to intracellular crystals, extracellular phytocrystalline deposits could not be reutilized (Fink, 1991a). By secreting CO in remote areas, such as the outer surfaces of the epidermis, the plant efficiently removed excess ions (Ca^{2+} and/or oxalate) from the internal tissues. Why some species formed intracellular *and* extracellular crystals was unknown. Conifers seemed to have evolved an additional mechanism for extracellular sequestration of CO as well as intracellularly in crystal idioblasts. Extracellular crystallization might eliminate the need to 'sacrifice' any cells as special crystal idioblasts (Fink, 1991a). Development of a crystalliferous cuticle might restrict herbivory by imposing mechanical constraints on larval feeding. Little predation was observed on *Casaurina* branchlets, which could be attributed to the CO crystals embedded in the cuticle (Berg, 1994).

Studies of Crystal Occurrence in the Genus *Dracaena*

The first to describe extracellular epidermal crystals was Kohl (1889). He reported relatively large rhombohedral crystals on a background of *Dracaena fragrans* epidermal cells (Figure 2-9). Dracaenas also exhibited intracellular CO

crystal deposition in the form of raphides. Scurfield and Michell (1973) determined that *D. fragrans* raphides consisted of the monohydrate form of CO. Fink (1991a) reported *D. marginata* as having small crystals embedded within the cuticular layer above the striated epidermal cell wall. *Dracaena sanderiana* deposited CO crystals in intracellular compartments and apoplastically between the outer epidermal cell wall and the cuticle (Vladimirova, 1996).

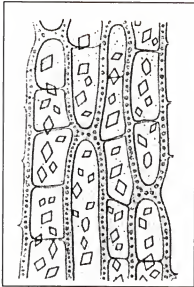


Figure 2-9. *Dracaena fragrans* crystals as seen by Kohl (redrawn from Kohl, (1889).

Summary and Conclusions

Biominingalization referred to the processes by which living organisms deposited minerals. Two types of crystal precipitation existed, namely: biologically induced and biologically controlled. Biologically induced deposition was characterized by minimal organismal control over the biominingalization process, and crystallization occurred without the presence of organic matrices. Biologically controlled deposition implied that the organism exerted structural, spatial, and chemical control over the mineral deposition, and organic matrices

were an integral part of the crystallization process. Controlled biomineralization offered an understanding of how organisms selected, localized, concentrated elements, and nucleated various minerals.

Species in more than 200 families of angiosperms and gymnosperms were shown to form CO crystals in various locations and quantities within their tissues. The two types of CO found in plants were COM and COD. CO crystals typically occurred as deposits in the vacuole or cytoplasm of highly specialized cells called crystal idioblasts. This mode of deposition was prevalent in angiosperms and was accompanied by the elaboration of a variety of subcellular structures, namely: cytoplasmic spherosomes, vacuolar organic paracrystalline bodies, membrane complexes, plasmalemmasomes, and crystal chambers. In contrast, coniferous gymnosperms have evolved a system of extracellular CO crystal deposition. This system was termed a crystalliferous cuticle if it occurred in the outer tangential cell walls of the epidermal cells. Very few angiosperms possessed extracellular crystal deposition, although differences between gymnosperm and angiosperm extracellular crystal precipitation did exist. The primary site of crystallization in the gymnosperms was believed to be the cell wall, whereas in the angiosperms evidence pointed to an intracellular origin and subsequent transport and encasement in the apoplastic space of the cell wall.

Very few studies have dealt with the monocotyledonous genus *Dracaena*, which possessed both cuticular epidermal and intracellular crystals. This study will characterize the chemical and crystallographic identity of the deposits, describe the extracellular crystal ontogeny, and determine the behavior of the various crystal types under different levels of Ca^{2+} supply. Characterization of

the cuticular epidermal crystals in selected species of *Dracaena* also was included.

CHAPTER 3 MATERIALS AND METHODS

General Tissue and Crystal Morphology

Cuticular Crystal Extraction and Processing

In order to separate the cuticular epidermal crystals from intracellular deposits, the following extraction procedures were followed. One gram of fresh mature leaf material was cut in 10x10 mm pieces and placed for 48 hrs in a 10 ml maceration solution containing cellulase (1.0% w/v), hemicellulase (1.0% w/v), and pectinase (0.1% w/v) (Protoplast Isolation Enzyme Solution I, Sigma). After maceration, adaxial and abaxial epidermal peels were obtained by gently pulling the epidermises away from the underlying mesophyll. The peels were rinsed three times in deionized water and dehydrated in a five-step (25%, 50%, 75%, 90%, and 100%) ascending ethyl alcohol series.

The isolated peels were separated into two groups. One group of isolated peels was ground to a fine paste in liquid nitrogen with a mortar and pestle. The goal was to simultaneously fracture the cuticle along planes of weakness, presumably along interfaces between crystals and cuticle/cell wall material, to release the crystals. The paste was used in two different procedures. The first procedure was preparation for X-ray diffraction analysis, which involved placing a small quantity of the paste on a glass slide and examining it with a Phillips-Norelco X-ray diffractometer using $\text{CuK}\alpha$ radiation at 40kV, 20 mA, and 10 min⁻¹ scan speed between 2θ from 20° and 60° . The results were

compared with the American Society for Testing Materials (ASTM) X-ray standards for calcium oxalate monohydrate (whewellite) and dihydrate (weddelite). ASTM data were obtained from the Joint Committee on Powder Diffraction Standards (JCPDS) – International Centre for Diffraction Data, 1996. The second procedure was preparation for SEM. The paste was resuspended in 100% ethyl alcohol and centrifuged at 10,000 rpm. Concentrated crystals were collected with a pipette from the bottom of the centrifuge tube, placed on a circular glass coverslip, mounted on aluminum stubs with carbon conductive paste, air dried, sputter-coated with Au in a IB-2 ion coater (Eiko Engineering), and finally examined with a HITACHI S-4000 SEM.

Demineralization Procedure

The second group of isolated epidermal peels was used for the observation of crystal tubercles (spaces occupied by the cuticular crystals) after removal of the cuticular crystals. The peels were placed in Trump's fixative (pH=7.2) (McDowell and Trump, 1976) for 18 hours at room temperature (20 °C), rinsed in PBS (Phosphate Buffered Saline, pH=7.2) followed by deionized water. They were then placed for 3 hrs in a demineralizing solution (S|P™ Decalcifying Solution, Baxter, Scientific Products). The solution contained EDTA, Sodium Potassium Tartrate, HCl, and Sodium Tartrate. During the procedure the solution was changed three times. After demineralization, the epidermal peels were rinsed twice in deionized water and dehydrated in a five-step ascending ethyl alcohol series, and critical point dried in CO₂. Sputter-coating and SEM were consistent with previous procedures.

Freeze Fracture of Epidermal Tissue

In order to facilitate observation of cuticular crystals *in situ*, mature *D. sanderiana* leaves were placed in Trump's fixative for 18 hours at room temperature, rinsed in PBS, postfixed for 1 h at 4 °C in 1% OsO₄ (same buffer), rinsed in PBS and deionized water, placed in 10% sucrose for 30 min, and cryofractured in liquid nitrogen. The fractured pieces were placed back in sucrose, dehydrated in a five-step ascending ethanol series, and critical point dried in CO₂. Sputter-coating and SEM were consistent with previous procedures.

Intracellular Crystal Extraction and Processing

Individual raphides were extracted by pressing freshly cut stems to circular glass coverslips and glass slides. The crystals were allowed to settle before the preparation was flooded in 100% ethanol, which was then drawn off with filter paper. The coverslips were prepared for SEM, and the glass slides were prepared for X-ray diffraction as outlined above. Intracellular deposits other than raphides were obtained from two sources, mature and immature leaves. Mature leaf pieces were placed for 24 hrs in a maceration solution containing cellulase, hemicellulase, and pectinase (see above). Intracellular deposits also were obtained from basal portions of developing leaf primordia (5-10 mm long), which also were placed in a maceration solution. The basal portions were used to minimize contamination from cuticular deposits. The basal portions were cut under an optical microscope equipped with polarizing optics to observe the cuticular crystals. The maceration procedure reduced all

internal tissue to individual cells and the epidermis to a long tube (due to the shape of a monocotyledonous leaf primordia). The epidermis was removed, and the cell suspension was pipetted on glass slides and circular glass coverslips. The glass slides and circular glass coverslips were examined under an optical microscope to determine the presence of any raphide contamination. Since few raphide bundles were present, a pure cell suspension was easily obtained. The suspension was then flooded with water, which caused protoplast swelling and cell rupture. The intracellular crystals were freed and came to rest at the bottom of the suspension. The excess water and cell debris were drawn off with filter paper. Three water rinses were followed by three 100% ethanol rinses. The coverslips were prepared for SEM, and the glass slides were processed for X-ray diffraction as outlined above.

Observations of Cuticular Crystals in Other *Dracaena* Species

Epidermal peels of *D. arborea* Willd., *D. deremensis* Engl., *D. draco* L., *D. fragrans* (L.) Ker-Gawl., *D. marginata* Lam., *D. massefiana* (a hybrid between *D. fragrans* cv. 'Massangeana' and *D. surculosa*), *D. reflexa* Lam., *D. surculosa* Lindl., and *D. thalioides* Hort. Makoy ex E. Morr. were isolated with procedures identical to the ones used on mature *D. sanderiana* leaves. All *Dracaena* species were grown in the Dept. of Environmental Horticulture conservatory (with the exception of leaf samples from *D. arborea*, which were donated from a local nursery). In addition, herbarium specimen of four species from the Jardin Botanico Canaria "Viera y Claijo" in Spain were examined: *D. cinnabari* Balf., *D. ellembeckiana* Hort. ex, *D. ombet* Kots. & Peyr., and *D. tamaranae* Marrero,

Almeida & Glez.-Martin. The first three species were grown in the Jardin Botanico Canaria "Viera y Claijo", while the last species originated from Gran Canaria. All species from Spain, *D. arborea* and *D. draco* belonged to the so-called Dragon Tree group. The epidermal peel isolation from herbarium specimens involved a 3 hour soak in boiling water prior to enzymatic digestion.

Optical and Transmission Electron Microscopies

Dracaena sanderiana shoot apices and mature leaf segments were fixed and postfixed as described for the freeze fracture procedure. After extended dehydration in a ten-step ascending ethanol series (deemed necessary due to the nature of the tissue), the samples were placed in a five-step ascending acetone series, and embedded in Spurr's low viscosity resin (Spurr, 1968). Thick (1 μ m) and thin (70-80nm) sections were cut on Reichert Supernova Ultramicrotome. All sections were cut transversely to the main axis of the shoot apex and the leaf. The thick sections were collected on glass slides and stained in 1% toluidine blue O buffered in borax (Feder and O'Brien, 1968). Thin sections were collected on Formvar-coated copper grids (100 mesh), stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined with a HITACHI H-7000 TEM. Epidermal peels obtained from fresh mature leaves, fresh shoot apices, macerated cells, and isolated crystals were observed with a Nikon Optiphot-Pol research microscope equipped with polarizing optics. Detailed cellular measurements were made with an ocular micrometer. Photographs were taken with an automatic Nikon UFX-II camera attachment.

Growth Experiments With Three Calcium Levels

Shoot apices with six leaves each were placed in leakproof containers in distilled water for 18 months in a greenhouse. During this time the cuttings formed roots and produced an average of four to five new leaves. The plants started showing signs of Ca deficiency (reduced leaf size and a reduced growth rate) after the first three months. However, they continued to appear otherwise healthy. The first six leaves that were on the cuttings when they were placed in water, dried out and were removed to deplete as much internal stored Ca as possible.

After eighteen months eighteen rooted cuttings were placed in 100ml beakers, each filled with 100 ppm of nitrogen (KNO_3) and 0, 3, or 7 mM Ca^{2+} . Nine of these cuttings were mineral-deficient and grown in the deionized water for 18 months (as described above). The other nine cuttings were non-deficient and obtained from healthy plants which had not been deprived of minerals. One mineral-deficient rooted cutting was placed in each of three beakers of each Ca^{2+} concentration, and one non-deficient rooted cutting was placed in each of three beakers of each Ca^{2+} concentration, for a total of 18 rooted cuttings in a completely randomized design (Figure 3-1).

Calcium acetate was used as the Ca^{2+} source because it has been shown to induce higher uptake of calcium ions in isolated leaflets of *Gleditsia triacanthos* compared to Ca-carbonate and Ca-lactate (Borchert, 1986). Other organic anions (citrate, glycolate, glyoxalate, malate) and inorganic ions (chloride, nitrate, sulfate) were prohibitive for Ca-uptake (Borchert, 1986). The acetate was reported as an "unlikely" candidate for the main substrate for

oxalate synthesis. The Ca concentrations (0, 3, or 7 mM Ca^{2+}) used in this study relied on previously reported experiments in which whole *Lemna minor* plants were induced to form intracellular raphides (Franceschi, 1989).

The beakers were covered with plastic film to minimize evaporation. Liquid level was kept at 80 ml with addition of distilled water. Plants were grown for four months in a growth chamber under 12 h photoperiod, radiation of $350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Photosynthetically Active Radiation, PAR) (light sources Sylvania VHO Full White lamps and Incandescent 25W rough surface lamps), and temperatures of 20/15 °C (day/night). Statistical analysis was performed using ANOVA procedure in Microsoft Excel®.

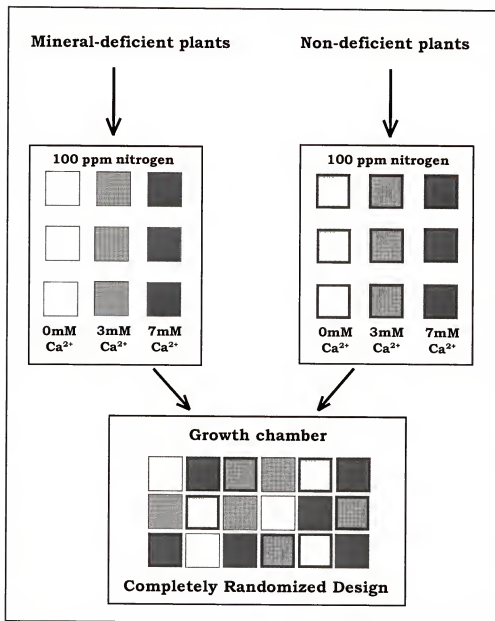


Figure 3-1. Schematic illustration of the experimental design.

CHAPTER 4

EXTRAPLASMIC CALCIUM OXALATE DEPOSITS IN *DRACAENA SANDERIANA*

General Crystal Morphology and X-Ray Diffraction Data

Cuticular crystals are best visualized when epidermal peels are examined with conventional light microscopy under crossed polars. Due to their anisotropic nature they are birefringent against the dark background of the epidermal cells (Figure 4-1A-C).

Epidermal cells of *Dracaena sanderiana* are elongated with length to width ratio ranging from 10:1 to 15:1. Adaxial and abaxial leaf surfaces are amphistomatic and possess identical epidermal layers with respect to the cells and crystals. Stomates are anomocytic with the guard cell pairs markedly different from the surrounding epidermal cells (Figure 4-1). Under low magnification the largest crystal deposits appear oriented along the long leaf/cell axis equidistant from the longitudinal cell walls (Figure 4-1A,C). The orientation of the long crystal axis is random, the largest deposits are somewhat equidistant from the longitudinal cell walls (Figure 4-1B). Guard cells are conspicuously crystal-free (Figure 4-1C). A thick cuticular layer obstructs the outlines of individual epidermal cells but diamond-shaped crystal tubercles are readily discernible (Figure 4-1D; Figure 4-2F).

SEM micrographs of freeze-fractured epidermal peels reveal numerous crystalline deposits attached to the cuticle underside (Figure 4-2A) with some crystals notably larger than the rest (Figure 4-2B).

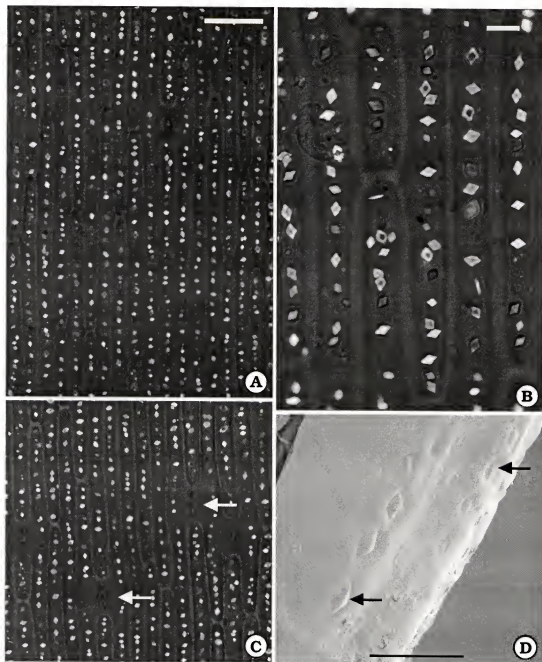


Figure 4-1. LM and SEM micrographs of surface features of *Dracaena sanderiana* epidermal peels.

A-C. Viewed in polarized light with crossed polars. Note that the larger epidermal crystalline deposits are oriented along the long leaf/cell axis equidistant from the longitudinal cell walls. Bar = 50 μ m.

B. Close-up of A. Bar = 10 μ m.

C. Note the absence of crystals in the guard cells (arrows). Bar = 10 μ m.

D. Crystal tubercles are clearly visible on the cuticle surface (arrows). Bar = 15 μ m.

Crystal polarity is frequently observed, i.e., an exposed rounded crystal face and flat, smoother side faces (Figure 4-2C). The crystal sides facing the cuticle almost invariably have a smooth texture (Figure 4-2E-G,I), while the crystal sides facing the leaf interior have a rough texture and rounded outlines (Figure 4-2B-C). A step-like layering is evident on the leaf interior-exposed crystal surface of some crystals (Figure 4-2H). The micrographs show that when crystals have evacuated an occupied place they leave behind a smooth impression in the cuticle but clearly outside the outer cell wall (Figure 4-2D).

X-ray powder diffraction (Table 4-1) and crystal morphology (Figure 4-3) confirm the identity of the cuticular deposits as calcium oxalate monohydrate (COM), or whewellite. The monoclinic COM form is characterized by a combination of planes, the most pronounced of which is $\{101\}$. Occasionally, the $\{010\}$ face also develops (Figure 4-3A,D). All isolated crystals show the typical form or variations of it (Figure 4-3B,C,E,F,H) achieved by the development of the additional planes $\{110\}$ and $\{011\}$. Multiple parallel lines intersecting $\{110\}$ planes are present in some crystals (Figure 4-3A,M). Successive concentric layers associated with particular crystal faces, especially ones facing the leaf interior, are exhibited by some crystals (Figure 4-3E).

When crystals are fractured during extraction, angular step-like layers are evident (Figure 4-3F). Crystals may display irregular shapes (Figure 4-3G) with poorly expressed or undeveloped crystal faces (Figure 4-3J-M). The size of the deposits ranges from less than $1\mu\text{m}$ to $8\mu\text{m}$ (Figure 4-4) when measured along the long crystal axis. Commonly the large crystals are $4\text{--}5\mu\text{m}$ long (Figure 4-3).

Table 4-1. Comparison of ASTM data of calcium oxalate monohydrate with crystals extracted from the foliar cuticle/epidermis of *Dracaena sanderiana*.

ASTM whewellite $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}^z$		<i>Dracaena sanderiana</i>	
D, Å ^y	I/I ₀ ^x	D, Å	I/I ₀
*5.93 ^w	100	5.93	100
5.79	30	-	-
*3.78	70	3.64	16.5
3.00	2	3.4	14.1
3.00	2	3.11	12.9
3.01	10	-	-
*2.97	45	2.97	53.9
2.92	10	-	-
2.84	10	-	-
2.00	4	2.53	3.8
2.49	18	-	-
2.36	30	-	-
2.35	12	-	-
2.21	6	2.21	7.9
2.08	14	-	-
1.98	10	1.98	9.8
1.95	10	-	-

^zASTM data were obtained from Joint Committee on Powder Diffraction Standards (JCPDS) – International Centre for Diffraction Data, 1996.

^yD is the wavelength spacings in Ångstroms.

^xI/I₀ is relative intensity of diffraction response for each analysis.

^wThe three major peaks are indicated by an asterisk (*) in each analysis.

Figure 4-2. SEM micrographs of *Dracaena sanderiana* cuticle.

A. Numerous small calcium oxalate monohydrate (COM) crystals cover cuticle underside (arrow, cuticle). Bar = 20 μ m.

B. Freeze-fracture exposes three large crystals situated in the cuticle. Bar = 6 μ m.

C. Close-up of the largest crystal in B. Note the texture of the exposed crystal faces. Bar = 1.7 μ m.

D. Crystal hole situated above the striated outer epidermal cell wall (down arrow, cuticle; right arrow, epidermal cell wall). Bar = 3 μ m.

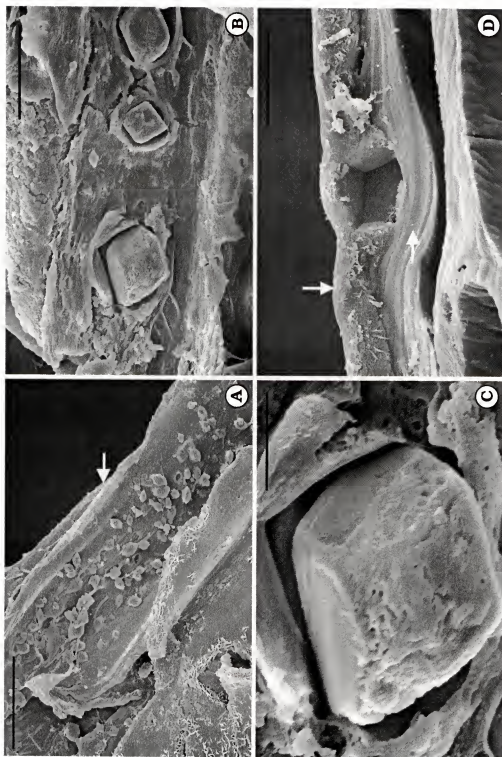


Figure 4-2 continued.

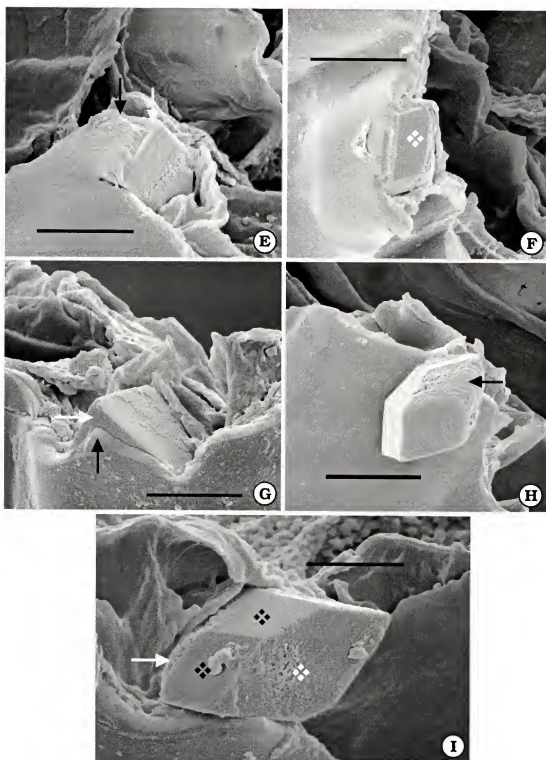
E and G. Note cuticle covering the crystal (black arrow) and the smoothness of the crystal face adjacent to the cuticle (white arrow).

F. Note the protuberance created by the crystal tubercle and the smooth exposed face (white star).

H. Note the step-like layering of the crystal face (arrow). This crystal has been displaced from its original location and now is facing the viewer with the side previously facing the leaf (compare with Figure 4-2B,C).

I. Note the exposed smooth crystal faces (stars). The side facing the cuticle is indicated by white star. Note that the opposite crystal face (arrow) has a rough texture. Bar = $3.75\mu\text{m}$.

E, F, G, and I. Bar = $3\mu\text{m}$.



Most crystals are isolated relatively unobstructed with foreign material, although some are enclosed in 'envelopes' (Figure 4-3I). The sharp crystal outlines are concealed in an amorphous envelope, which has relatively smooth surfaces (Figure 4-3I). A part of the envelope may be still attached to the crystal (Figure 4-3J-K). In some light micrographs the crystal envelope appears as a thin line surrounding the crystal (Figure 4-4A). Under polarized light the thin light line is isotropic. Crystal envelopes are readily observed after the crystals have been removed by demineralization (Figure 4-4B-E). The envelopes consist of two parts: 1) an outer amorphous to granular material, likely compacted cuticular waxes (Figure 4-4D); and 2) an inner thick, smooth layer adhering to the crystal with different degree of tenacity (Figure 4-3I-K; Figure 4-4E). The cuticle surrounding the crystal envelope appears compacted, or pushed away (Figure 4-4G). In instances where two crystals develop in close proximity, a common envelope wall is present between them (Figure 4-4H-I).

Crystal Development

The cuticular crystals appear very early in leaf ontogeny (Figure 4-5) and first appear in a primordium approximately 1.5-2% of mature leaf size.

Dracaena sanderiana possesses an involute vernation and umbricate aestivation that results in each primordium encircling the next youngest one. During early developmental stages, the leaf primordium resembles an elongated cone (Figure 4-5A-D). As a monocotyledonous species, *D. sanderiana* displays acropetal leaf maturation. After early primordial development, nearly all growth in the leaf occurs through activity of a basal intercalary meristem and thus the oldest portion of the leaf is toward its distal end.

Figure 4-3. SEM micrographs of isolated cuticular COM crystals.

A-D. Numbers in brackets identify crystal faces and represent indices of Miller planes.

A. A typical form of a monoclinic COM crystal with expressed $\{\bar{1}01\}$ and $\{010\}$ planes. Note parallel lines (white arrow) evident on $\{110\}$ planes.

B and C. Variations on the same crystal form without the $\{010\}$ plane.

A-D. Bars = 1.7, 2, 3 and 1.7 μm , respectively.

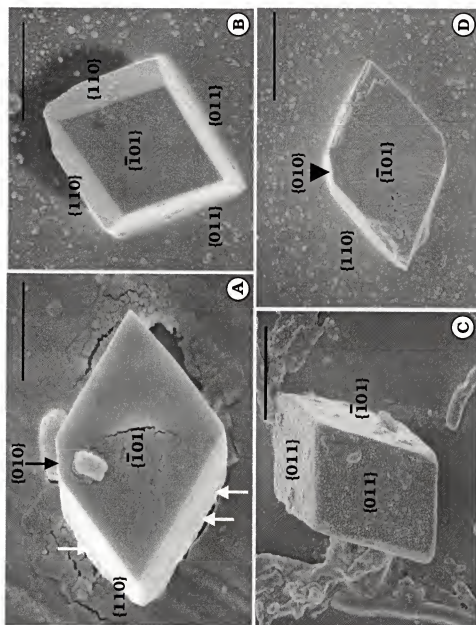


Figure 4-3 continued.

E. Note rounded $\{\bar{1}01\}$ face and step-like layers (arrow).

F. The $\{110\}$ planes (arrows) appear much smaller in this particular crystal. Note angular layering on the fractured faces (white arrow).

G. The crystal is truncated along $\{\bar{1}01\}$ and $\{011\}$ planes.

E-H. Bars = 1.5, 2.3, 2.3, and 2 μm , respectively.

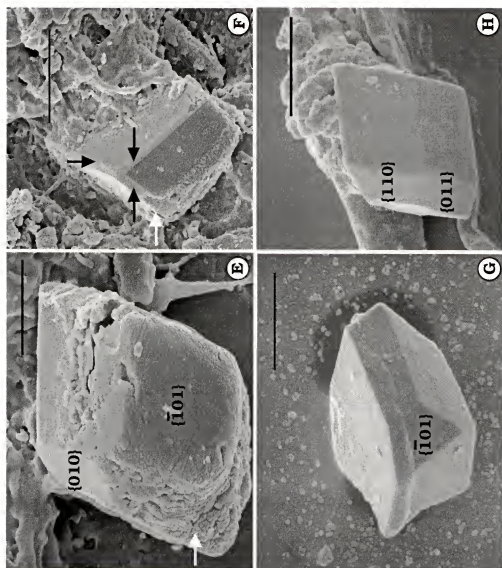


Figure 4-3 continued.

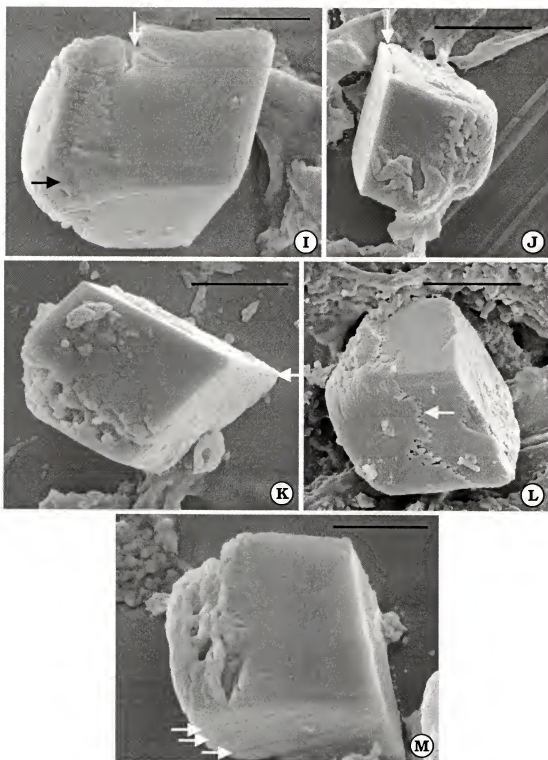
I. The crystal is isolated with its crystal envelope, which is torn (white arrow). Note rounded outlines of the envelope (black arrow). Bar = $1.2\mu\text{m}$.

J. One side of the crystal envelope is still attached to the crystal (arrow in J and K). Bar = $2\mu\text{m}$.

K. Arrow points at crystal envelope still attached to the crystal. Bar = $1.5\mu\text{m}$.

L. Amorphous material partially covering the crystal is visible (arrow). Bar = $2\mu\text{m}$.

M. Note parallel lines (arrows) on crystal face identical to the ones in A. Bar = $1.2\mu\text{m}$.



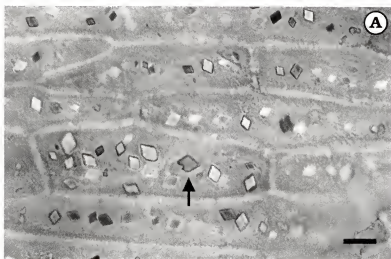
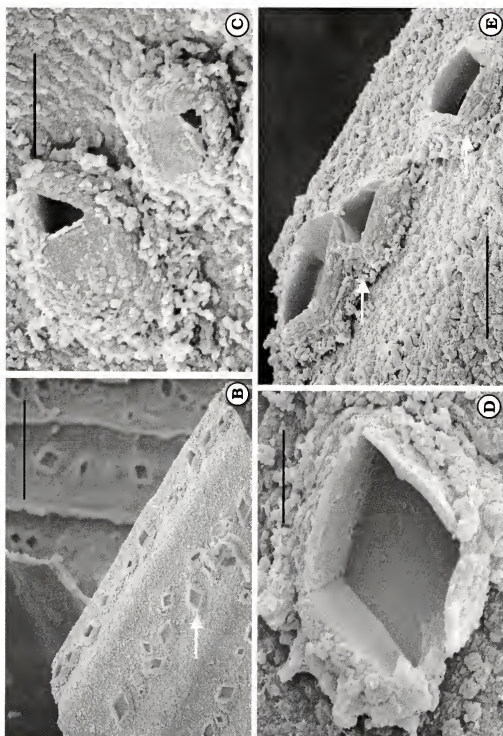


Figure 4-4. LM and SEM micrographs of epidermal peels of *Dracaena sanderiana*.

A. Crystal envelope is seen as a lighter-colored line around two crystals (arrow). Bar = 10 μ m.

Figure 4-4 continued.

- B. Underside of the demineralized cuticle shows crystal envelopes devoid of crystals (arrow). Bar = 15 μ m.
- C. Close-up of two empty envelopes. Note granular to amorphous material surrounding the envelopes. Bar = 1.7 μ m.
- D. Close-up of a crystal envelope. Bar = 1.5 μ m.
- E. Amorphous crystal envelope material (arrows). Bar = 3 μ m.



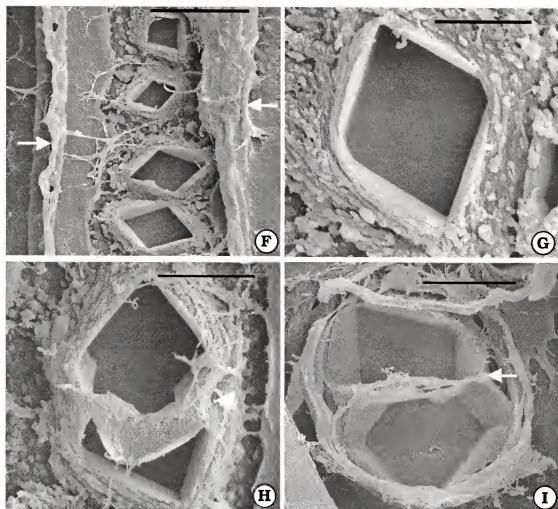


Figure 4-4 continued.

F. The smooth material is cell wall remnants. Note the granular amorphous cuticle underneath the cell wall. Note that the large crystals are situated between the two epidermal cell walls (arrows). Bar = $5\mu\text{m}$.

G. Note the smooth outlines of the crystal envelope and the compressed amorphous material around it. Bar = $1.5\mu\text{m}$.

H. A common envelope wall (arrow) separates two intergrown crystals. Bar = $2.3\mu\text{m}$.

I. Similar to H with less material in the common crystal envelope wall (arrow). Bar = $3.3\mu\text{m}$.

Crystal development proceeds naturally in accordance with this mode of leaf tissue maturation. When the leaf primordium is approximately 600 μ m in length (Figure 4-5A-B), no evidence of cuticular or intracellular crystalline deposits is observed. As the primordium continues to grow and elongate, cellular differentiation and maturation occurs and intracellular COM is deposited in the form of raphide bundles (Figure 4-5C-D). Raphide bundles form earlier than cuticular crystals. The latter are not detectable until the primordium reaches approximately 2500-3000 μ m in length (Figure 4-5C). They are first observed in the epidermal cells of the leaf tip. In general, there is a "crystal maturation zone" (Figure 4-5D). Over an area of approximately 300-500 μ m (Figure 4-5D,F-H; Figure 4-6A-B), cuticular COM crystals appear in developing epidermal cells that have reached approximately 7-10% of their mature length (Figure 4-6A-B). Initially, two to five crystals per cell are visible. As cells continue to mature, more crystals form per cell (Figure 4-6C). At this early stage of their growth, all deposits appear rod-like and they are distributed randomly with respect to the long cell axis. The length to width ratio of most epidermal cells at this stage is 3:1 to 4:1. Crystals continue to grow, and the largest ones reach final size (Figure 4-5D) before the epidermal cells have reached their final length.

Some crystals deposited in the same cell's cuticle reach maximum size, while others are comparatively small (Figure 4-6I). The guard cells that differentiate at the same time do not form cuticular crystals (Figure 4-6F). As the epidermal cells reach their maximal size, the largest crystals are lined up along the midline and seemingly form a single crystal file (Figure 4-6G).

Figure 4-5. LM micrographs of crystal development in *Dracaena sanderiana* shoot apex.

A-H. Whole mounts of shoot apices viewed in polarized light with partially crossed (A and B) and fully crossed polars (C-H). Note: the designations 'first', 'second' and 'third' leaf primordia are in reality progressive developmental stages of one leaf primordium as it elongates. The designations are used for simplicity.

A. Focus on the dome-shaped meristem (arrow). The bright outline is a residual birefringence, not related to crystals.

B. Focus on the first primordium (arrow). Note the absence of any crystalline deposits at this developmental stage. Bar = 100 μ m.

C. Composite micrograph of the second leaf primordium. At this stage the primordium is an elongated cone. Note the acropetal maturation of the cuticular epidermal crystals and the raphide bundles which form earlier (arrow). Bar = 150 μ m.

D. Close-up of crystal maturation zone of the second leaf primordium depicted in C. Bar = 50 μ m.

E. Composite micrograph of the third leaf primordium. Note extensive cuticular crystal deposition. Due to an increase in overall tissue thickness and numbers of cuticular deposits, the birefringence of earlier formed raphides (toward the leaf tip) is obscured. Bar = 200 μ m.

F-H. Close-up of the crystal maturation zone, depicted in E. Bar = 50 μ m.

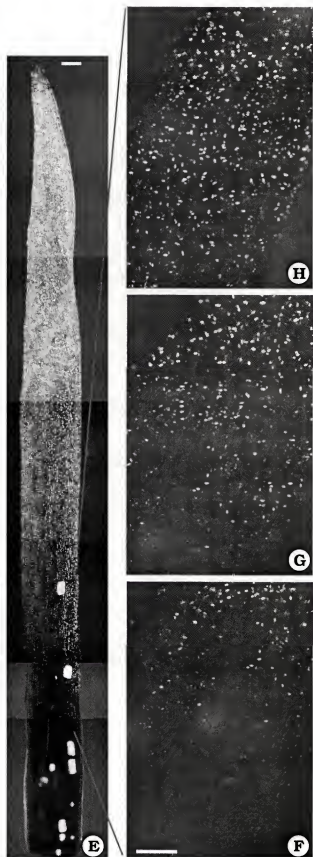
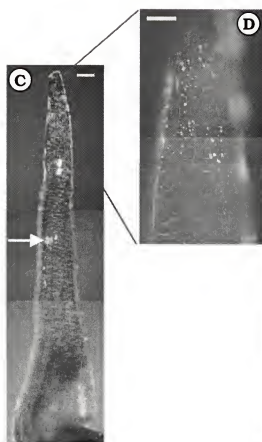
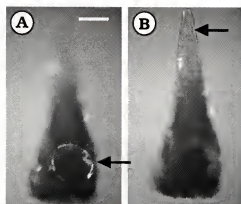


Figure 4-6. LM micrographs of the second leaf primordium in *Dracaena sanderiana*.

A-D. Close-up of the crystal maturation zone as seen in Figure 4-5F-H.

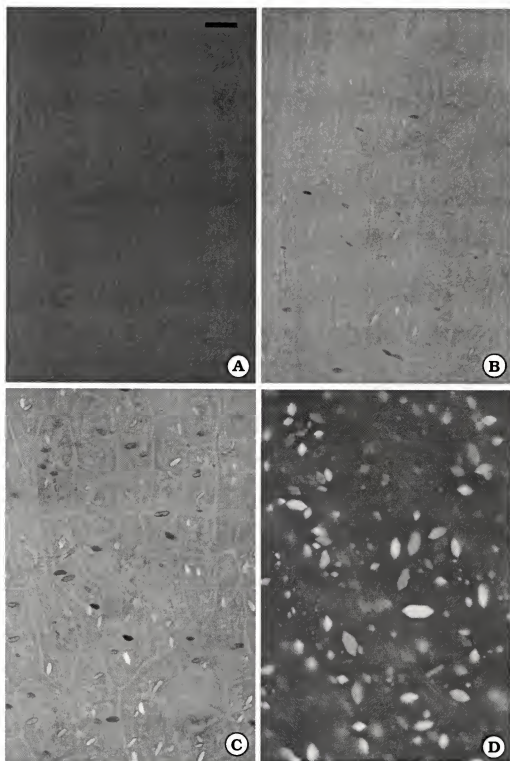
A and D. Polarizer and analyzer are completely crossed; B and C, partially crossed polars. The partial crossing assists in visualizing the underlying epidermal cells.

A. No crystals are evident. Bar = 10 μ m.

B. Crystals begin to appear. The epidermal cells are elongating but have not yet reached their final length.

C. More rod-like crystals appear. The crystal colors are related to the orientation of the crystal axis with respect to the polarizer and analyzer, e.g., dark-blue crystals are extinct, bright-yellow crystals show maximum birefringence.

D. As more crystals appear, the largest ones reach maximum size and a shape typical of COM.



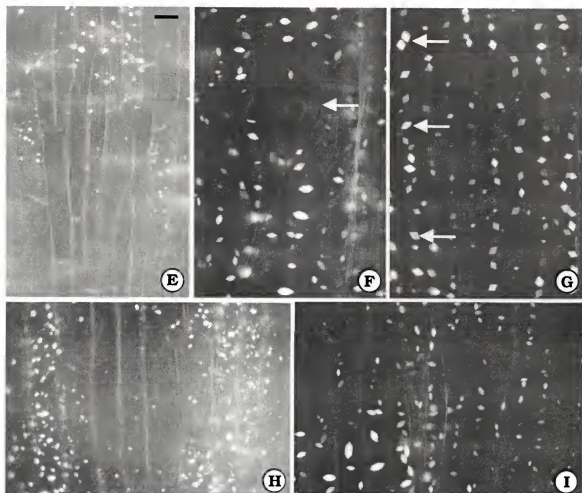


Figure 4-6 continued.

E. Crystals are not formed uniformly in epidermal cells of similar age.
Bar = 10 μ m.

F. Crystals are absent from the pair of guard cells (arrow).

G. Largest crystals are situated along the midline of the epidermal cells and form a single file parallel to the longitudinal cell walls of the epidermal cell (arrows).

H. Some epidermal cells may be devoid of any large crystals at this stage.

I. Crystals do not grow uniformly with respect to cell age.

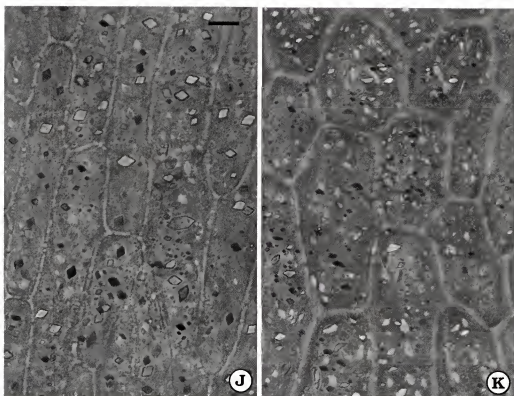


Figure 4-6 continued.

J. Epidermal cells just below the leaf tip. Bar = 10 μ m.

K. Epidermal cells at the tip of leaf primordium. Note the differences in cell shape and crystal size.

Figure 4-7. LM micrographs of *Dracaena sandariana* leaves.

1-3. Low magnification LM micrographs of progressive transverse sections through the length of the shoot apex showing positions of leaf primordia with respect to each other and the meristem. All sections were cut in transverse plane relative to the main axis of the shoot apex. M, meristem; LP1, first leaf primordium; LP2, second leaf primordium; LP3, third leaf primordium. Bar = 100 μ m. Note that at this particular stage LP1 is the size of LP3 in Figure 4-5; therefore the first and second leaf primordia in Figure 4-5 are not present in Figure 4-7.

A. Transverse section at the base of the second leaf primordium and the meristem. Bar = 10 μ m.

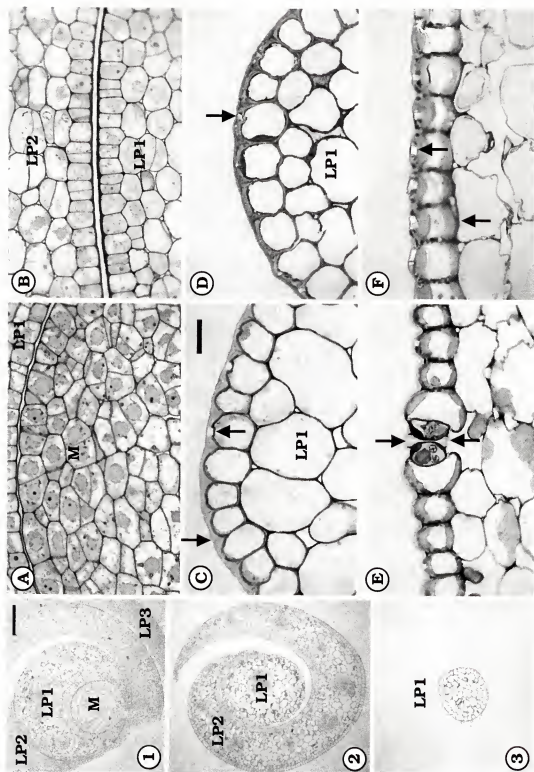
B. Transverse section of the first (LP1) and second (LP2) leaf primordia approx. 200 μ m from the meristem. Crystals are not evident at this stage in either primordia. Note the increase in thickness of the cuticle compared to A.

C. Transverse section of the first (LP1) approx. 3000 μ m from the meristem. Cuticle continues to increase in thickness (white arrow) and crystals become evident (black arrow).

D. Transverse section of the first (LP1) approx. 5000 μ m from the meristem. Crystals increase in size and number (black arrow) and the outer tangential epidermal cell wall continues to increase in thickness.

E. Transverse section of a mature leaf. Note the absence of crystals in the cuticle above the guard cell pair (up black arrow) and the prominent cuticular ledges (down black arrow).

F. Transverse section of a mature leaf. Note greatly thickened outer and inner tangential epidermal cell walls (arrows). Large COM crystals are present above every epidermal cell.



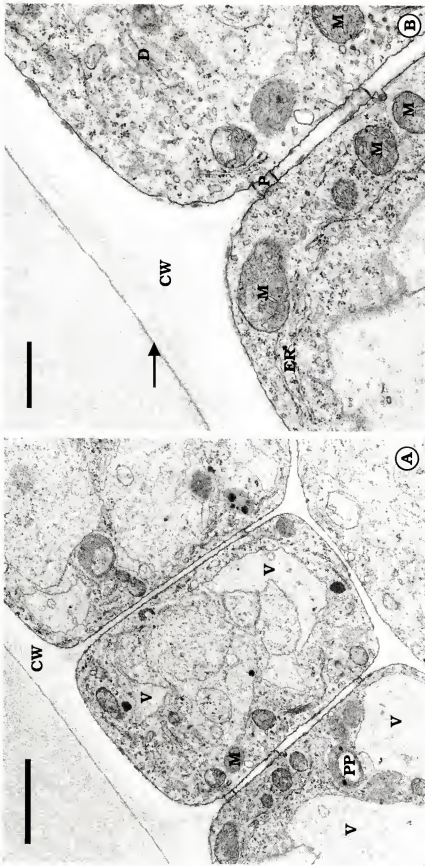


Figure 4-8. TEM micrographs of immature epidermal cells in *Dracaena sandertiana* leaf primordium.

A. Low magnification view of epidermal cells in developing leaf primordium. Bar = 2 μ m.

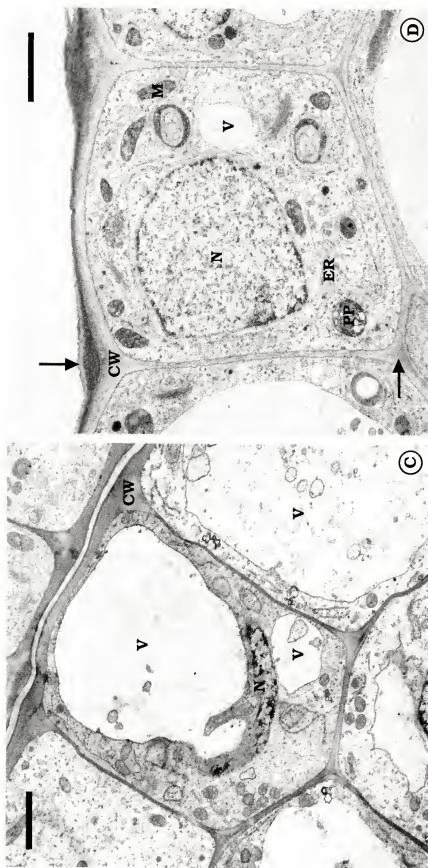
B. High magnification view of A. Cuticle is a very thin layer (arrow). Crystals are not present at this stage. Note the two plasmodesmata between the epidermal cells. Bar = 0.7 μ m.

Figure abbreviations: C, cuticle; CC, crystal chamber; CH, crystal hole; CW, cell wall; D, dictyosome; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; P, plasmodesmata; PP, proplastid; RER, rough ER; V, vacuole; VS, vesicle.

Epidermal cells distal from the leaf tip (Figure 4-6J) exhibit a higher length to width ratio when compared to cells that are proximal to the leaf tip (Figure 4-6K). Crystals that developed in the former cells are larger and appear more or less oriented parallel to the long cell axis. In contrast, cells proximal to the leaf tip display smaller, randomly distributed crystals.

As indicated earlier, *D. sanderiana* possesses an involute vernation and umbriate aestivation that results in each primordium encircling the next youngest one. As a leaf primordium elongates, its shape changes from circular (proximal to the tip) to crescent-shaped (proximal to the base) in transverse section (Figure 4-71-3). Development of cuticular COM crystals was examined using leaf primordia approximately 5% of mature size (Figure 4-7). Young meristematic cells at the leaf base do not possess crystalline deposits discernible with polarized light microscopy (Figure 4-7A), and these deposits are not observed when the cuticular layer becomes evident (Figure 4-7B). They are first detectable in the region where the epidermal cells are completely contiguous (Figure 4-7C).

Distally in a leaf primordium, crystals become more numerous (Figure 4-7D), until in a mature leaf, COM crystals occur beneath the cuticular layer in every epidermal cell (Figure 4-7E-F). Ultrastructurally, developing *D. sanderiana* primordium reveal that immature epidermal cells at the base of the primordium are roughly rectangular in transverse section (Figure 4-8A-D), and possess a thin cell wall which is thicker in the outer regions and especially at the junction between two epidermal cells (Figure 4-8C). Plasmodesmata are common between epidermal cells (Figure 4-8B).



Proplastids with well-developed starch grains and electron-dense plastoglobuli are present at this early growth stage (Figure 4-8A,D). The cytoplasm features free ribosomes and short profiles of endoplasmic reticulum (ER) in association with ribosomes. The vacuolar apparatus is represented by numerous pleiomorphic structures of various sizes and number per cell. Some cell vacuoles appear electron-lucent, while others are filled with smaller vesicles and amorphous or fibrillar material (Figure 4-8C). Mitochondria with well-developed cristae are abundant especially near the cell wall.

Cuticular development is initiated approximately 200-300 μ m from the leaf primordium base (Figure 4-8B). A mature leaf cuticle of *D. sanderiana* is composed of epicuticular wax, the cuticle proper, and a cutinized layer, pectin layer, and cellulose layer. This structural layering is particularly evident in the region over an anticlinal junction between two epidermal cells (Figure 4-9A) and at the leaf margins (Figure 4-9B). The cuticle proper consists of strongly osmiophilic opaque lamellae of variable thickness that alternate with lucent ones of more uniform thickness.

The epicuticular wax on the outside of the polylamellate region is an electron-lucent zone marked by a thin superficial deposit of osmiophilic material. The cutin layer features peculiar amorphous circular structures, or "cutin balls" (Lyshede, 1982) (Figure 4-9A). The pectin layer and the cellulose layer exhibit uniform homogeneity.

Immature epidermal cells possess paramural bodies that occur most commonly proximal to the cell wall (Figure 4-10), and often release their contents directly into the apoplastic space (Figure 4-10A,C-F).

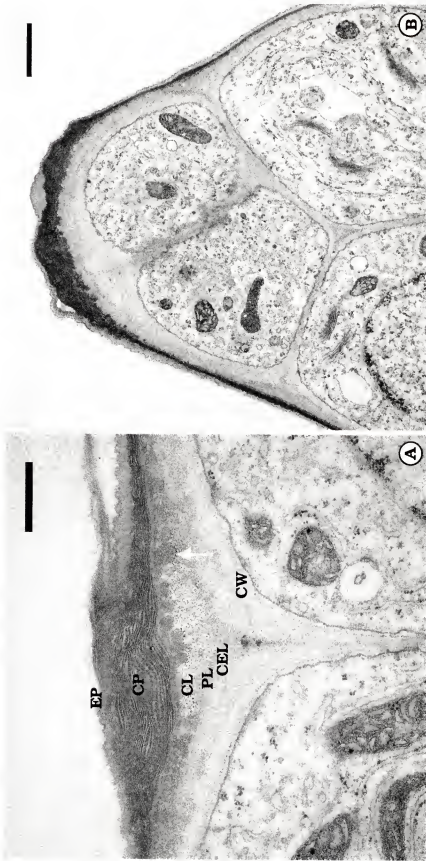


Figure 4-9. TEM micrographs of cuticle development in leaf primordium of *Dracaena sandertiana*.

Note: the cuticular layer is described in detail with reference to its structural components (terminology after Lyshede, 1982); in all other figures 'cuticle' is used as an inclusive term.

A. Cuticular components: EP, epicuticular wax; CP, cuticle proper; CL, cutinized layer; PL, pectin layer; CEL, cellulose layer. Arrow, 'cutin balls'. Bar = 0.5µm.

B. Epidermal cells at the leaf margin feature greatest cuticle thickness. Bar = 1µm.

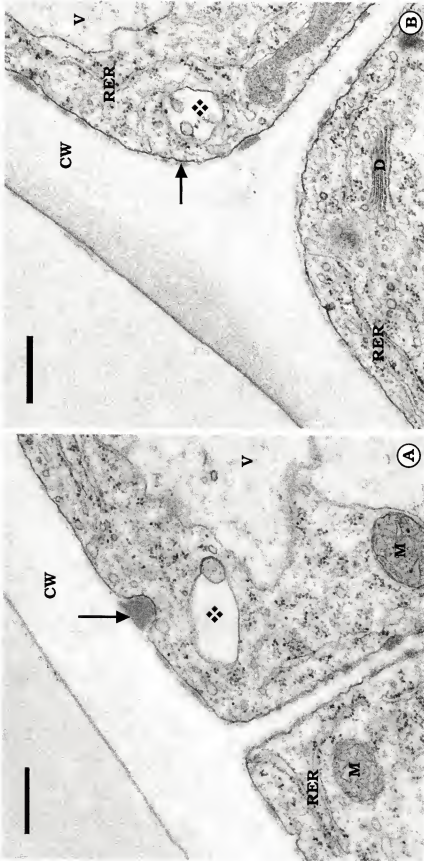


Figure 4-10. TEM micrographs of paramural bodies in immature epidermal cells of *Dracaena sandertiana*.

A. Paramural body with a round outline (star) is seen in the cytoplasmic space in close proximity to the plasmalemma (right black arrow in B) and the vacuole. The paramural body is slightly elongated with a vesicle in the polar position. Note the extensive cytoplasmic ribosomes. Bar = 0.5 μm.

B. The paramural body (star) has a round shape and contains two small vesicles near its boundary. Note the short profiles of rough ER between the paramural body and the plasmalemma. Bar = 0.5 μm.

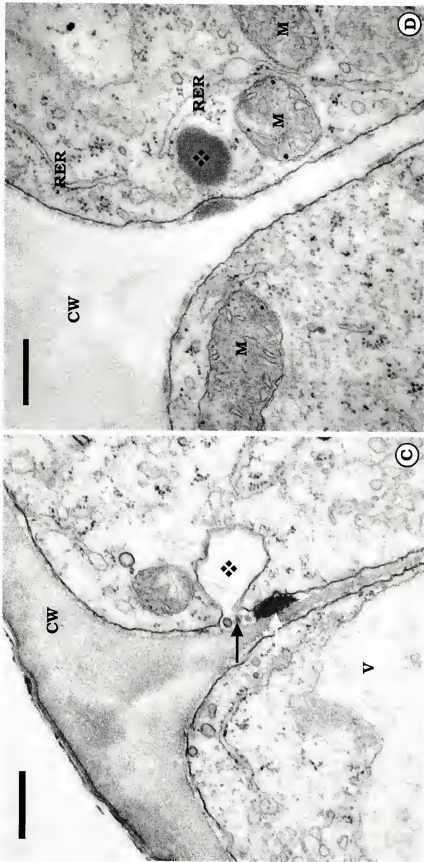


Figure 4-10 continued.

C. Paramural body, likely a plasmalemmasome (star), features a narrow orifice and three smaller vesicles (black arrow). White arrow, a homogeneously electron-dense body. Bar = 0.5 μ m.

D. A homogeneously electron-dense body, likely a lipid droplet, (star) is seen in transition from the cytoplasm through the plasmalemma to the cell wall (see also black arrow in A, and white arrow in C). A membrane cannot be discerned surrounding the lipid globules. Short profiles of RER are also present in close proximity. Bar = 0.3 μ m.

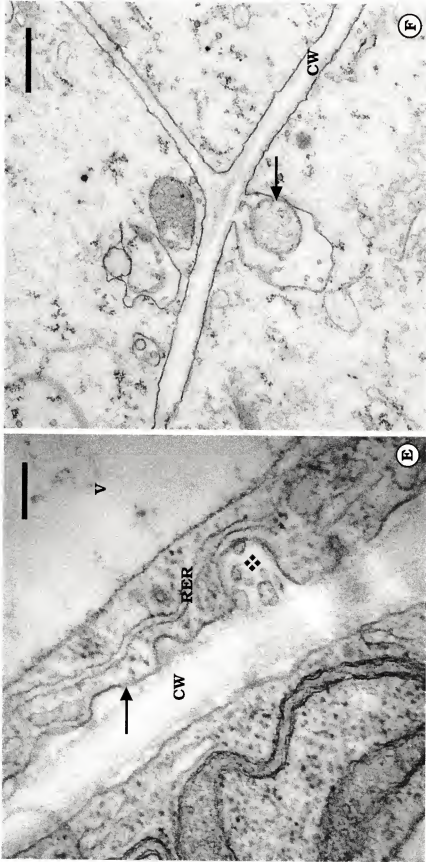


Figure 4-10 continued.

E. Paramural body (star), similar to the one depicted in C. Several small electron-dense vesicles are being released into the cell wall space. Note undulate plasma membrane (arrow). Bar = 0.2 μ m.

F. Larger paramural body (arrow) containing a single vesicle bounded by double membrane. Contents of the vesicle appear similar to the surrounding cytoplasm. Bar = 0.7 μ m.



Figure 4-11. TEM micrographs of subcellular structures in immature epidermal cells of *Dracaena sandertiana*.

A. Myelin figures located in the central vacuole. Bar = 0.4 μ m.

B. Structures made of concentrically-layered endoplasmic reticulum. Less electron-opaque layers alternate with denser ones (down arrow) and enclose an electron-lucent central part. Homogeneous layers associated with ribosomes, also encircle an electron-lucent portion (left arrow). Bar = 2 μ m.

Paramural bodies have contents with either a very low electron density (Figure 4-10A-C) or a density similar to that of lipid droplets (Figure 4-10D). The density and homogeneity of the contents, however, differ from that of cytoplasm. Some paramural bodies have internal vesicles (Figure 4-10A,B,C,D,E) which may have a double membrane (Figure 4-10F). In instances when the paramural bodies are found near the vacuole and the cell wall, short profiles of ribosomal ER are observed in very close association with them (Figure 4-10A,E). A sinuous plasma membrane with invaginations in association with the paramural bodies is observed (Figure 4-10F).

Myelin figures, concentric layers and short profiles of fibrillar material are observed in the vacuoles of some developing epidermal cells (Figure 4-11A). A distinctive cytological feature of the epidermal cells is a concentrically-layered ER (Figure 4-11B-D). These ER constructs occupy as much as 50% of the epidermal cell volume (Figure 4-11B,D), and differ in contents. Some constructs have dense electron-opaque layers alternating with less dense ones, while others appear homogeneous with granular layers in association with ribosomes (Figure 4-11B). Other structures display loose multiple concentric layers of rough ER that enclose a central part with cytoplasm (Figure 4-11C-D). The concentrically-layered ER is involved in formation of intracellular vesicles (Figure 4-11C). A single cisterna of rough ER, extending from the stacked portion and joining a profile of concentric RER is connected to a large, round vesicle in close association with the central vacuole (Figure 4-11D).

Immature epidermal cells of *D. sanderiana* are characterized by prominent Golgi apparatus that consists of numerous dictyosomes and associated structures (Figure 4-11E-F).

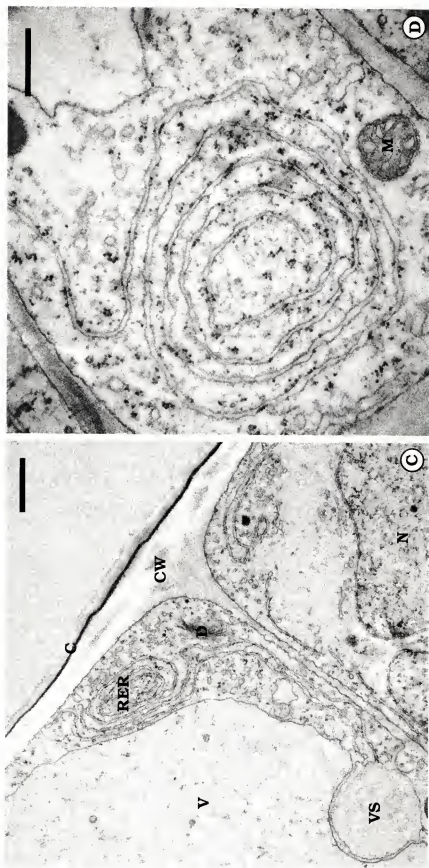


Figure 4-11 continued.

C. Concentrically layered rough ER connected to a large vesicle (VS). Bar = $1\mu\text{m}$.

D. Concentrically layered rough ER occupying a large portion of the cell. Bar = $0.4\mu\text{m}$.

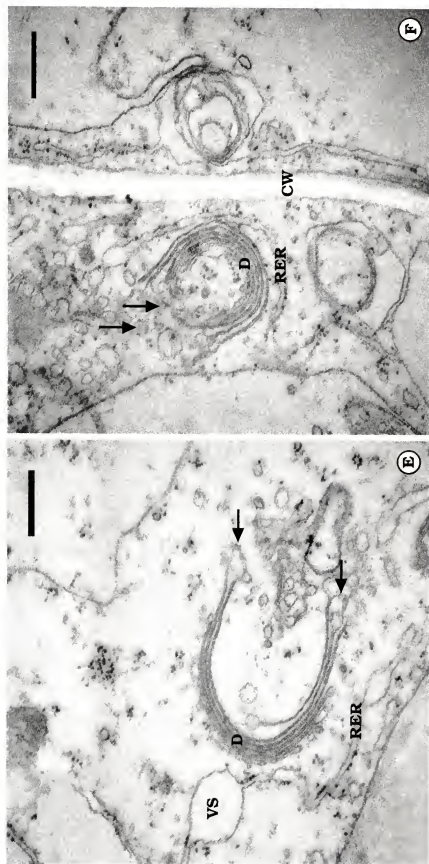


Figure 4-11 continued.

E. Dictyosome displaying vesicle formation at the *cis* and *trans* sides (arrows). Small vesicle connected to RER is evident.

F. Dictyosome displaying enhanced vesicle formation (arrows) at the medial side.

E and F. Bar = 0.5 μ m.



Figure 4-12. TEM micrographs of developing crystals in immature *Dracaena sandertana* epidermal cells.

A. Note highly invaginated plasma membrane (up arrow) and the presence of an electron-dense vesicle to the outside of the cell (down black arrowheads). Similar electron-dense vesicle is apparent in B. Bar = 0.5 μ m.

B. Rectangularly-shaped crystal chamber is likely derived from coalescence of smaller vesicles. Note variations in vesicle shape and an increase in thickness of the cuticular layer from A to B. Bar = 0.4 μ m.

Dictyosomes are large and frequently clustered, and dilations of the cisternae are common (Figure 4-11E). Relatively large vesicles with electron-transparent contents are produced by encapsulation of medial dictyosomal portions (Figure 4-11F).

The initial event in the deposition of cuticular COM crystals is the development of a highly plicate plasma membrane (Figure 4-12A). The folds are very conspicuous and appear to segregate the cytoplasm. Highly pleiomorphic vesicles (approx. $0.1\mu\text{m}$ in diameter) with electron-dense and electron-transparent contents are in close association with the plasmalemma. A rectangularly-shaped crystal chamber arises (approx. $0.5\mu\text{m}$ in diameter) from coalescence of the vesicles (Figure 4-12B). An electron-dense structure, the crystal chamber, is present in a section parallel to the crystal (Figure 4-12E). This chamber is situated in a fold or a space previously occupied by the cytoplasm, its outer boundary is clearly delineated by the immature cell wall. Lamellate larger vesicles (approx. $0.3\mu\text{m}$ in diameter) of various shape are initially observed internally (Figure 4-12C) and later externally in close proximity to the growing crystal (Figure 4-12D).

Crystals can protrude in the epidermal cell space in early growth stages (Figure 4-12G-H). Crystal shapes in transverse section vary but most typically are rectangular (based on the shape of the crystal holes). The electron-dense crystal chambers are not readily discernible in late stages of crystal growth.

As crystals continue to grow, they exert an inward pressure and occupy larger areas in the epidermal cell space (Figure 4-12I-J). The cuticle and/or the nascent cell wall do not allow for expansion of the crystals in an outwardly direction.

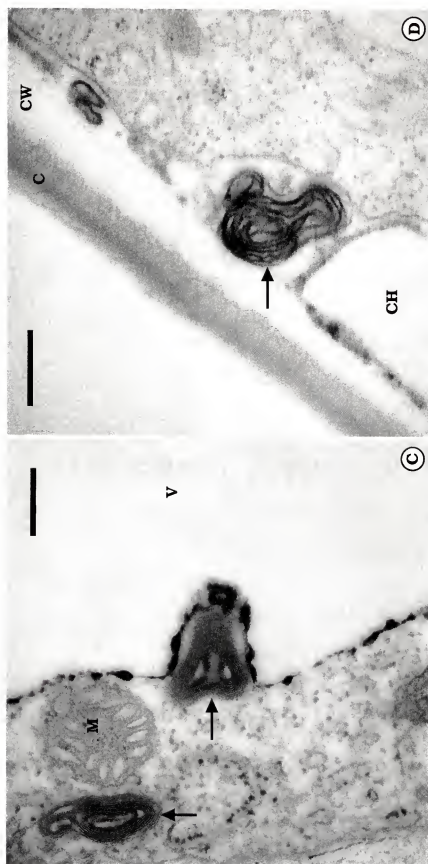
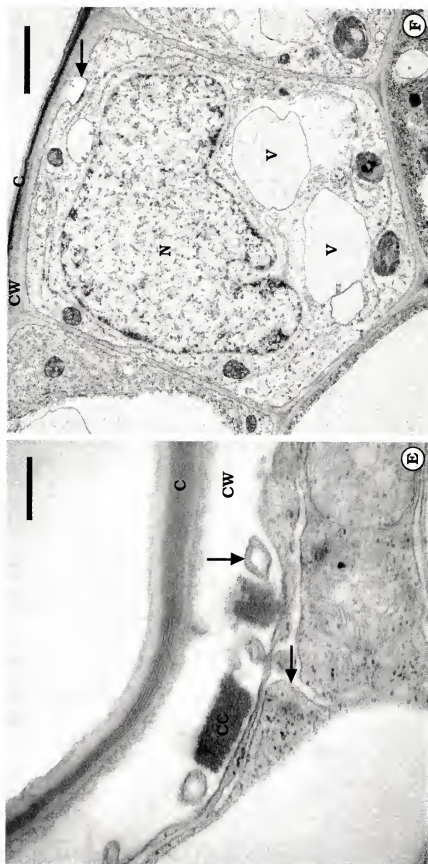


Figure 4-12 continued.

C. Lamellate vesicles (arrows) are initially observed in the cytoplasm in proximity to the vacuole. Bar = 0.2 μ m.

D. Lamellate vesicles are later observed outside of the cell in proximity to a developing crystal. Bar = 0.3 μ m.



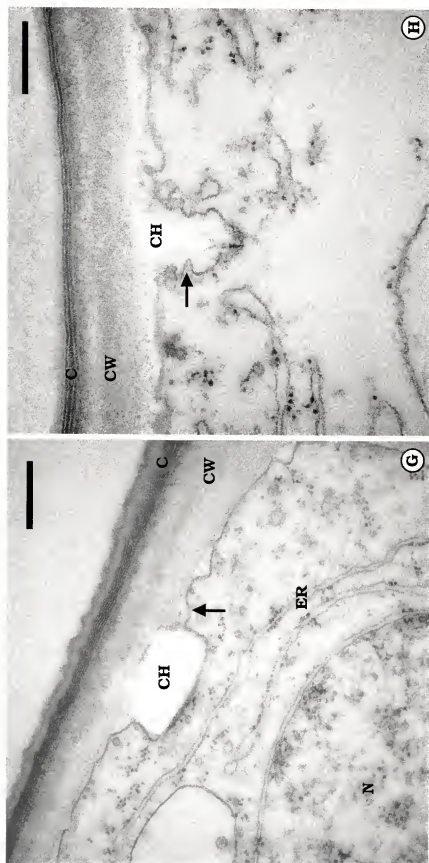
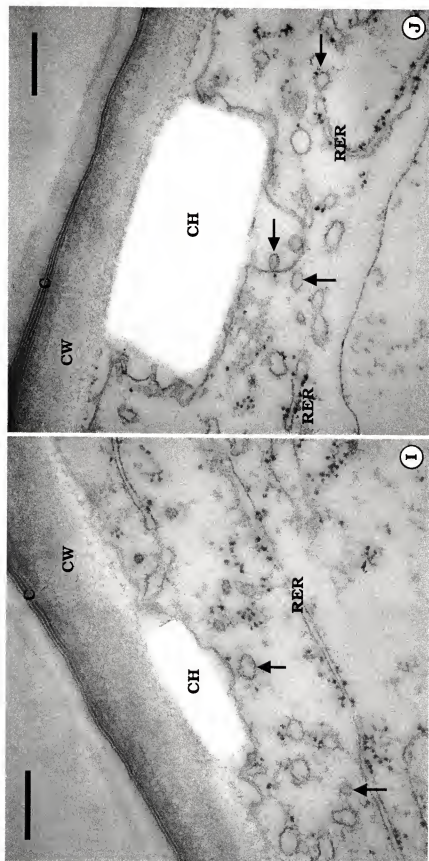


Figure 4-12 continued.

G and H. In the early stages of growth, crystals can protrude well into the epidermal cell space. Note invaginated plasmalemma (arrows). Bars = 0.4 and 0.3 μm , respectively.



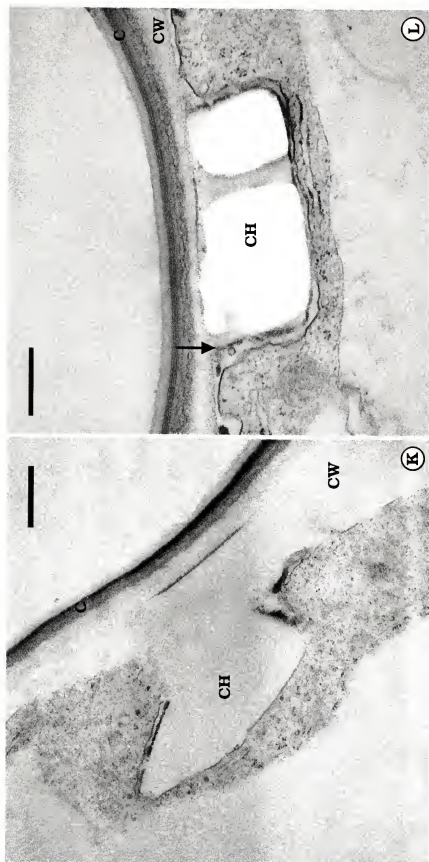


Figure 4-12 continued.

K and L. As crystals continue to grow, they enlarge at the expense of underlying cytoplasm. Note crystal chamber membrane (arrow) is visible between the growing crystal and the cytoplasm. Bars = 0.7 and 0.5 μ m, respectively.

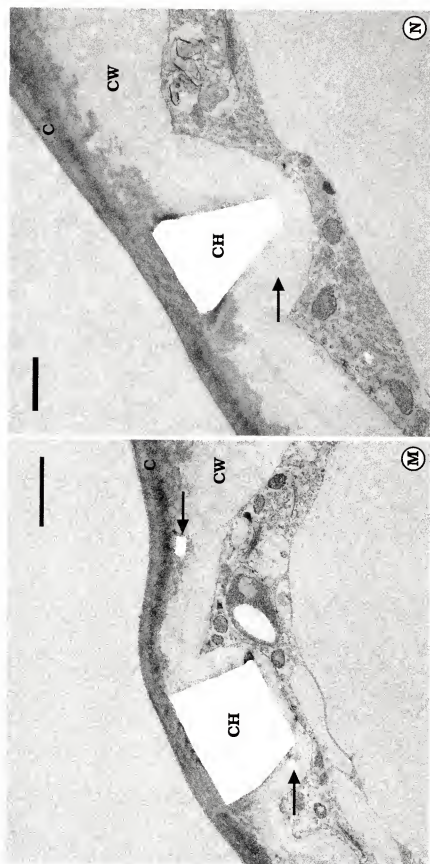


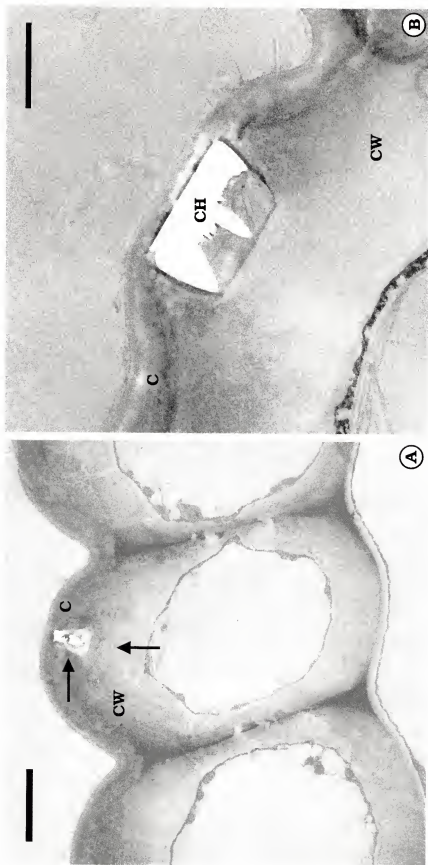
Figure 4-12 continued.

M and N. Thickening cell wall (right arrows) begins displacing the crystal away from the epidermal cell. At this stage the crystal has reached its final size. Note the small crystal beneath the cuticle (left arrow). In M, the crystal has pushed the cuticle upwards, while in N there is no evidence of such strain. Bars = 2 and 1 μ m, respectively.

Figure 4-13. TEM micrographs of *Dracaena sandertiana* mature epidermal cells.

A and B. In mature cells, extraplasmic crystals are removed from the cytoplasm and are closer to the cuticle. A marked indentation in the outer cell wall layers around the crystal is evident (up arrow). The crystal is embedded partially in the cuticle (right arrow). Note striated outer epidermal cell wall. Bar = 5 μ m.

B. The crystal has been pushed outwards and a protuberance is present on the leaf surface. Bar = 0.5 μ m.



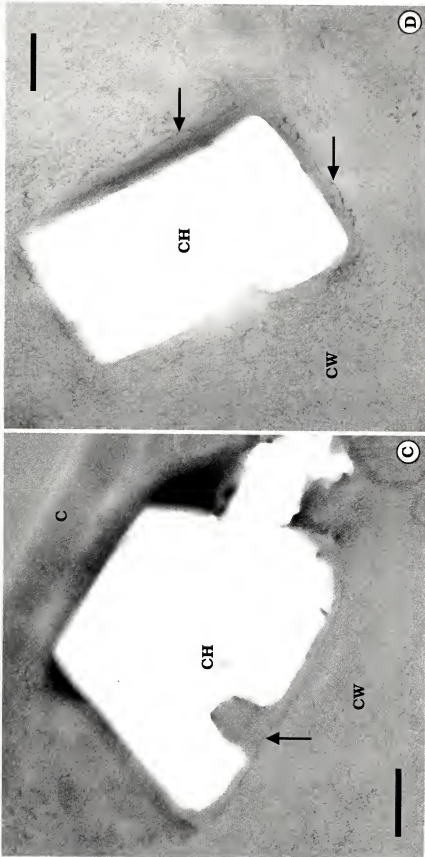


Figure 4-13 continued.

C and D. Note crystal chamber, seen as a thin line (arrows) around the crystals. White space to the right of the crystal hole in C is a tear in the section. Bars = 0.5 and 0.2 μ m, respectively.

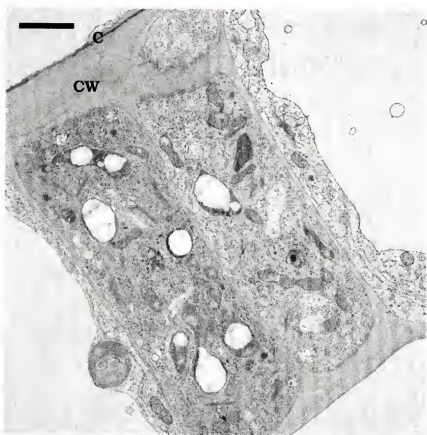


Figure 4-14. TEM micrograph of an immature guard cell pair in a developing leaf of *Dracaena sanderiana*. Bar = 2 μ m.

Small electron-lucent vesicles (approx. $0.1\mu\text{m}$ in diameter) traverse the cytoplasm (Figure 4-12I) and occur on the outside of the plasmalemma in the vicinity of the crystal (Figure 4-12J). The vesicles appear intimately associated with long profiles of RER (Figure 4-12J).

As the crystals continue to develop, they protrude deeper into the cytoplasmic space (Figure 4-12K-L). The shape of the crystal chambers in transverse section is variable. At this stage the deposits have reached approximately $2.5\mu\text{m}$ in length, but the outer tangential epidermal cell wall has not started to thicken yet. A space occurs between the enlarging crystal and the plasma membrane (Figure 4-12L), begins to widen as the epidermal cell wall increases in thickness (Figure 4-12M). Crystals are pushed away from the cytoplasm as they enlarge and obtain final size (Figure 4-12N). The space previously occupied by the deposit remains as an indentation in the epidermal cell wall (Figure 4-12L).

The outer tangential wall of the epidermal cells is thicker than the inner one, and the radial walls are thinner than both tangential walls in the mature cell (Figure 4-13A). Layering and crystal indentation in the outer wall are preserved as the cellulose layers are deposited. Portions of the cuticular layers occur around some crystals (Figure 4-13A), but not around other ones (Figure 4-13B).

The largest COM crystals develop in areas equidistant from the radial epidermal cell walls in transverse section (Figure 4-13A-B). The crystal chamber is evident as a thin line surrounding each cuticular crystal (Figure 4-13C-D). The chamber is amorphous in composition and differs from the fibrillar nature of the cellulose matrix of the cell wall.

Figure 4-15. LM, SEM, and TEM micrographs of extraplasmic COM crystals in mature epidermal and mesophyll cells of *Dracaena sandertiana*.

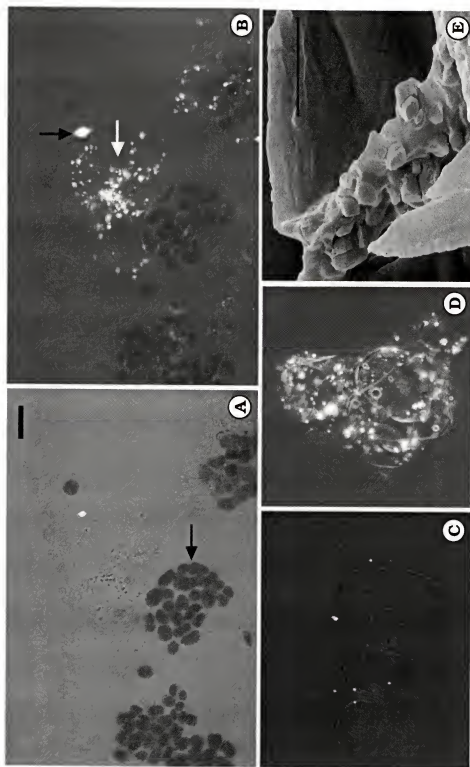
A-D. Macerated mesophyll cells. B-D. Viewed in polarized light with crossed polars and a gypsum plate (C).

A. Chlorophyllous mesophyll cells (arrow) appear crystal-free.

B. Achlorophyllous mesophyll cells display numerous small extraplasmic crystals. Note the larger free crystal (black arrow) which is a cuticular COM.

C-D. Amount of extraplasmic crystals differs among achlorophyllous cells.

E. Large quantity of small crystals are attached to the radial epidermal cell walls. A-E. Bars = 10µm.



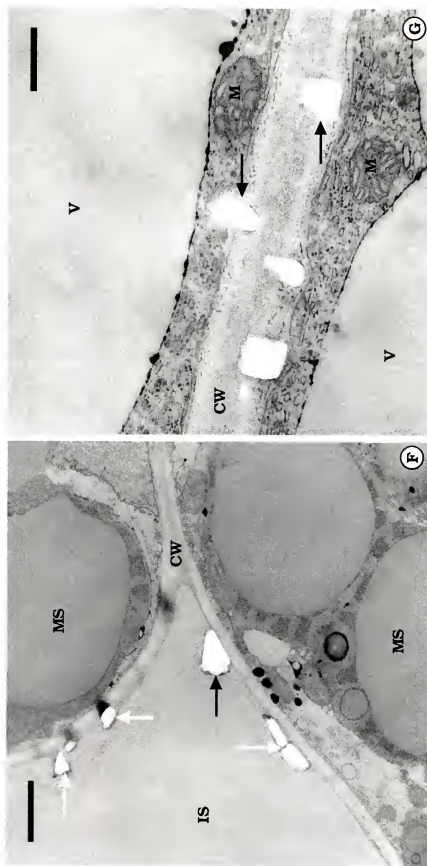


Figure 4-15 continued.

F. Extracellular crystals are attached (black arrow), partially embedded (right white arrow) in the cell wall facing intercellular spaces (IS) in the mesophyll. Note the thin electron-dense layer covering the two crystals (down white arrow). Bar = $2\mu\text{m}$.

G. Small crystals between mesophyll cells (MC) with no intracellular space available, are more (left black arrow) or less (right black arrow) closer to the cell cytoplasm. Bar = $0.4\mu\text{m}$.

Guard cells differentiate from meristematic initials before deposition of cuticular COM crystals is initiated (Figure 4-14). The guard cell pair does not exhibit any extraplasmic crystal activity and remains crystal-free, even after the neighboring epidermal cells have completed their ontogeny and crystal deposition.

In addition to extraplasmic crystals in the cuticular/apoplastic space, *D. sanderiana* possesses large quantities of minute crystals in the extracellular spaces internal to the leaf tissues (Figure 4-15). These deposits are of extraplasmic origin and are best visualized after complete maceration of the leaf mesophyll. Large chlorophyllous cells derived from the spongy mesophyll appear free of extraplasmic deposits attached to their cell walls (Figure 4-15A), whereas smaller, achlorophyllous cells have varying numbers of extracellular crystals (Figure 4-15B-D). Most crystals are considerably smaller in comparison to the cuticular COM deposits (Figure 4-15B); however, their strong birefringence and shape are indicative of COM (Figure 4-15E).

The internal extraplasmic crystals can be attached to, partially embedded, or fully embedded in the mesophyll cell wall (Figure 4-15F). A thin layer of electron-dense material covers the crystals even when they are barely attached to the cell wall (Figure 4-15F).

In the area of cell walls facing intercellular spaces crystals are more or less attached to the cell wall, whereas in regions where two cells abut the crystals are embedded in the cell walls (Figure 4-15G). In the latter instances the deposits occur proximal to the mesophyll cell plasmalemma.

Discussion

Crystal Morphology and Patterns of Deposition

Initially the orientation of cuticular crystals in *D. sanderiana* appear highly consistent with respect to the long axis of the leaf. The epidermal cells are very elongated and arrayed in linear files and the cuticular deposits follow similar linear patterns although most of the crystals do not show any preferred orientation of their long axis with respect to the long leaf axis. They are somewhat equidistant from the longitudinal cell walls when the shapes of epidermal cells distal (Figure 4-6J) and proximal to the leaf tip (Figure 4-6K) are compared.

Of special interest is the lack of crystals in the cuticular space above the guard cells. Conflicting reports exist regarding the presence or absence of apoplastic crystals associated with guard cells. While analyzing the cuticular characteristics of Cupressaceae, Alvin et al. (1982) reported that in *Callitris endlicheri* "the guard cell cuticle was pitted by embedded CO crystals". However, Borchert (1984) did not find CO crystals in the stomatal apparatus of *Gleditsia triacanthos*, a species which reportedly deposits crystals abundantly in cuticular areas. The lack of information on cuticular crystal deposition precludes generalized statements concerning the importance of this phenomenon. Clearly the deposition of cuticular COM crystals is not a part of the developmental sequence in the guard cells of *D. sanderiana*, which strongly suggests some controlling mechanism. Whether the internal 'machinery' for depositing cuticular CO is not present in the guard cells, ion flow is more

strictly regulated, or some other explanation accounts for the lack of crystals remains to be determined.

Calcium ions are known to play a crucial part in signal transduction in stomatal guard cells (Blatt and Thiel, 1993). Under elevated rhizospheric Ca^{2+} levels, specialized subsidiary cells in *Commelina communis* exhibited varying CO crystal deposition while the guard cells remained crystal-free (Ruiz and Mansfield, 1994). Conceivably, the stomatal complex in *D. sanderiana*, which lacks specialized subsidiary cells, functions in a similar fashion and exerts strict control over Ca^{2+} concentration in guard cells.

The presence of cuticular crystals on both leaf surfaces as well as over the entire plant body is intriguing since such a pattern has not been reported before in seed plants. *Casaurina* forms CO crystals in the abaxial epidermal cell walls but not in the adaxial epidermal cell walls (Berg, 1994). How such varying patterns of crystal deposition are achieved is enigmatic.

The present study is the first attempt to isolate and characterize cuticular crystals in a plant species, and also is the first conclusive report on the chemical nature of the deposits. The hydration state of the cuticular CO is expected since most reported studies of such crystals have alluded to the COM form. Their morphology is similar to the typical COM crystals grown synthetically (Sikes and Wierzbicki, 1996). Expression of the typical faces $\{101\}$, $\{010\}$, $\{110\}$, and $\{011\}$ has been previously observed in intracellular plant crystals and described in detail by Cody and Horner (1984). Of interest is the absence of twinning crystals in the present study, which may be indicative of specific conditions required for this phenomenon. Slow evaporation rates at room temperature yielded monocrystalline growth of COM (Cody and Horner, 1984).

An increase in the single/twin ratio also was caused by the presence of calcium chelators such as citric acid, ethylene diaminetetracetate (EDTA), and biotin and oxalate complexes such as iron (Vasilikiotis et al, 1983). Insoluble and lipid substances floating on the liquid surface decreased evaporation rate and increased monocrystalline formation.

The existence of multiple parallel lines intersecting $\{110\}$ planes in some crystals (Figure 4-3A,M) is especially intriguing. The explanation could be as simple as cleavage planes (special planes in a crystal along which the bonds between atoms of different layers are weaker), or as complex as intracrystalline inclusions of unknown nature (Parkinson, pers.comm.). Intercalation of organic material on specific planes different from the cleavage planes often occurs in biogenic minerals (Addadi and Weiner, 1989). The existence of intracrystalline proteins that have been intercalated inside single crystals along precise crystallographic orientations during crystal growth is a likely explanation for the modified characteristics of biologically-derived minerals (Addadi and Weiner, 1989). Plant crystals treated with proteases exhibit various degrees of dissolution and indicate the presence of proteins inside their structure (Parkinson and Fleming, pers. comm.).

The observed polarity in surface texture of some crystals with respect to their cuticular orientation is interesting (Figure 4-2B-C,E-G,I). The successive concentric layers and the rough texture associated with the leaf interior-exposed crystal faces (Figure 4-2H) could be features that are indicative of crystalline matter precipitated under the influence of a biological organism in a membrane-bound sheath (Lowenstam and Weiner, 1989). The existence of crystal envelopes supports such an interpretation. If the plant exerts some

influence over crystal nucleation and growth, the identity of the participating substances and the mode of their action are yet to be determined.

Crystal deposition in *D. sanderiana* occurs precociously when the leaf primordium is approximately 2% of mature leaf size. Such early development of crystals has been reported in *Beta vulgaris* where CO crystal sand deposition was initiated in a leaf less than 10% of final size (Franceschi, 1984). The onset of cuticular COM deposition in the developing leaf primordium is preceded by deposition of CO in the form of intracellular raphide idioblasts. Although in general a "crystal maturation zone" exists, crystal deposition does not proceed uniformly in all cells. Epidermal cells could remain crystal-free after neighboring cells (at identical developmental stages) have initiated crystal deposition (Figure 4-5E,H). Such a mode of deposition can be related to local patterns of ion availability. Borchert (1990) found that Ca^{2+} acted as a developmental signal in the formation of CO crystal spacing patterns in *Carya ovata*. After large crystals were formed, Ca^{2+} was depleted from the apoplastic space. This prevented new crystals from being formed in the vicinity of the large ones. The very high single/twin ratio of the cuticular COM crystals could also be related to localized ion concentration zones.

Growing cuticular crystals display morphological characteristics that are illustrated in Figures 4-16-18. Clearly, cuticular COM shapes (Figure 4-6B-D), and the crystal shape depicted in Figure 4-16, are very similar. Two growing cuticular crystals and an underlying epidermal cell are shown in Figure 4-17 (compare with Figure 4-6B-D). The plane of *b* and *c* axes in both crystals is parallel to the long axis of the epidermal cell/leaf, while the *a* axis is perpendicular.

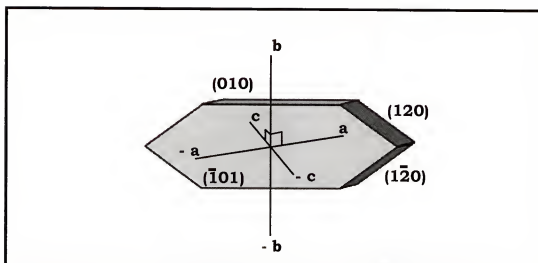


Figure 4-16. Schematic illustration showing orientation of the three crystal axes and principal crystal faces in developing cuticular crystal from the epidermis of *Dracaena sanderiana* (crystal morphology after Sikes and Wierzbicki, 1996).

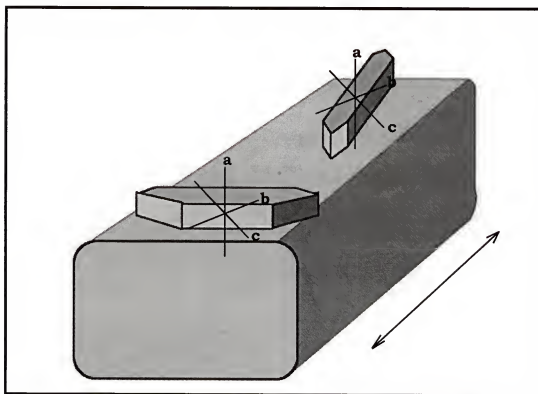


Figure 4-17. Schematic illustration showing orientation of the three crystal axes of two growing COM crystals with respect to the epidermal cells and the leaf. Note that the plane of *b* and *c* axes is parallel to the long axis of the epidermal cell/leaf, while *a* axis is perpendicular. Arrow on the right indicates direction of cell elongation. Compare with Figure 4-6F-G. Not drawn to scale.

Such preferential orientation of the crystallographic axes has been reported for CaCO_3 tablets in mollusk species, and could provide insights into the nucleating sites themselves (Lowenstam and Weiner, 1989). For example, the aragonite crystals (CaCO_3) in gastropod nacre show no preferential alignment of their b and a axes with respect to each other, but the c axes are well aligned. This alignment means that adjacent crystals are randomly rotated about their c axes with respect to each other. Similarly, cuticular COM crystals display random rotation about their a axes with respect to their neighbors. Information regarding alignment of nucleating sites in the epidermal cells of *D. sanderiana* can be inferred with knowledge about nature of the organic matrix where the COM crystals nucleate. In biomineralization a specific mineral can be produced with defined size and orientation due to the chemistry and geometry of the initiation site. Heuer et al. (1992) suggested that nucleation can be regulated via one of three ways, namely: periodic, negatively charged surfaces; bifunctional scaffolding molecules; and epitaxial elements with a critical number of sites for nucleation.

Additional information about growth of cuticular COM crystals is shown in Figure 4-18. The rhombohedral (diamond) shape is achieved by faster growth of $\{010\}$ faces with respect to the other crystal faces. Crystals that have reached final size are characterized by very well expressed $\{\bar{1}01\}$ faces and much smaller $\{010\}$ ones.

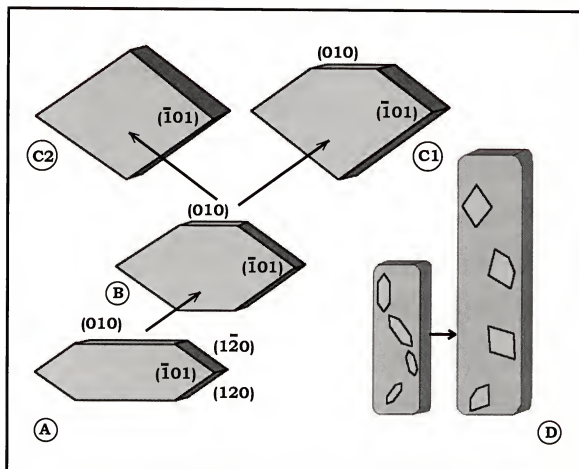


Figure 4-18. Schematic illustration showing a likely growth sequence (A through C) of the cuticular COM crystals in *Dracaena sanderiana*.

A. Principal crystal faces ($\bar{1}01$) and (010) are present.

B. (010) faces are displaying faster growth compared to (120) faces.

Note: growth along other planes is not shown, in reality the crystals show growth along all planes in order to reach final size. The diagram is used to illustrate relative growth of (010) planes with respect to other crystal planes.

C1. One (010) face has grown itself out of existence, while the other (010) face is still present, albeit smaller compared to B.

C2. Both (010) crystal faces have disappeared.

D. Changes in crystal shape as seen in surface view in the epidermal cells of *Dracaena sanderiana*. Compare with Figure 4-6F-G. Not drawn to scale.

To gain a better understanding of the crystallization phenomenon, the following questions should be addressed (the list adopts some questions posed for biomineralization in marine organisms (Lowenstam and Weiner, 1989)):

What are the stages of assembly of the organic membranes, which are part of the crystal chambers in *D. sanderiana* epidermal cells?

What is the precise distribution of individual macromolecules on the membrane surface?

What are the size and the structure of the nucleation sites?

What is the structure of the crystal surface overlying the nucleation site?

Do membrane components become intercalated in the COM crystals?

Is there a difference in the constituents of the crystal chamber present at the 'top' (cuticle side) and the 'bottom' (leaf side)?

Is cessation of crystal growth brought about by lack of ion supply to the mineralization site?

What determines the hydration state of the CO crystals?

The answers to these questions remain to be determined.

Ultrastructural Elements in Epidermal Cells and Their Relationship to Crystal Deposition

Cuticle development in *D. sanderiana* was evaluated and described using terminology adopted from Lyshede (1982). All five layers, epicuticular wax, cuticle proper, cutinized layer, pectin layer, and the cellulose layer are present. *Dracaena sanderiana* cuticle can be categorized as Type I as defined by Halloway (1982), and is very similar in structure to the cuticle of *Agave americana* and other xerophytic plants.

Invaginations of the plasmalemma is a recognized developmental feature in the major groups of organisms (Mahlberg et al., 1971). Paramural bodies are classified as plasmalemmasomes derived from the plasmalemma, or lomasomes derived from cytoplasmic membranes (Marchant and Robards, 1968). These organelles are thought to be involved in wall synthesis, either as a transitory stage during the incorporation of wall precursors, or as the site of incorporation of enzymes for extracellular synthesis.

Origin of the paramural bodies in *D. sanderiana* could not be established unequivocally, although they are observed both in the cytoplasm and the plasmalemma region. Both sites indicate transport of paramural bodies from the cell's interior to the apoplast. Plasmalemmasomes have been reported previously in developing crystal idioblastic cells (Mollenhauer and Larson, 1966; Horner and Whitmoyer, 1972; Horner and Wagner, 1980; Horner et al., 1981; Horner and Wagner, 1995). However, all reports of such occurrences have been with reference to intracellular crystals. Plasmalemma invaginations occur in the epidermal cells of several aquatic angiosperms such as *Potamogeton*, and have been proposed as the locations of H^+ efflux in the apoplast (Prins and Helder, 1985). In these plants the plasmalemmasomes were found only in the epidermal tissue on the side of the leaf where HCO_3^- is absorbed.

Myelin figures, found in algae, fungi, and higher plants, have had several functions suggested for them, namely: cytoplasmic alteration (Carbonel and Polak, 1962) and wall formation (Wilsenach and Kessel, 1965; Essau et al., 1966). However, they also have been listed as artifacts due to the failure of aldehyde fixation to stabilize lipids. The extent of myelin figures observed in a

given tissue is thought to be partly a function of the lipid composition of the tissue (Schneeberger et al., 1976).

Concentric aggregates of rough endoplasmic reticulum (RER) have been found in several plant cells in organs such as resting buds (Shih and Rappaport, 1971), excised roots (Morriset, 1983), seed coats (Yaklich et al., 1992), and incompatible pollen grains (Wales and Han, 1998). The formation of concentric ER structures in these cells generally is considered to be a response to stress conditions such as anoxia and plasmolysis (Morriset, 1983) and is considered a reversible process. Longitudinal stacking of RER has been reported in mature pollen grains and presumed to represent an inhibition of protein synthesis (Cresti et al., 1985). The presence of concentrically-layered RER in *D. sanderiana* is not easily explained in light of the above interpretations, although similar layering has been reported in crystal idioblasts of *Vanilla planifolia* and *Monstera deliciosa* (Mollenhauer and Larson, 1966). Abundant ER, particularly in the plasmalemma region has been reported in crystalliferous epidermal cells of *Casaurina* (Berg, 1994) and in developing astrosclereids of *Nymphaea* (Kuo-Huang, 1992).

The predominant features of *D. sanderiana* immature epidermal cells are paramural bodies with variously electron-dense contents, an abundance of RER, large quantities of cytoplasmic ribosomes, and clusters of dictyosomes with many associated vesicles. This diverse array of subcellular structures in meristematic epidermal cells of *D. sanderiana* is highly suggestive of a very dynamic system similar to many crystal idioblasts (Franceschi and Horner, 1980b). Thus, an epidermal *D. sanderiana* cell, which deposits apoplastic crystals resembles a crystal idioblast, which deposits intracellular crystals.

Initial events in the deposition of cuticular COM crystals were the development of a highly plicate plasma membrane and numerous pleiomorphic and lamellate vesicular bodies in the general region facing the cuticle (Figure 4-12). After vesicle fusion, rectangularly-shaped crystal chambers with electron-dense structures emerged. These electron-dense structures are not always present in later stages of crystal growth and possibly become a part of the 'crystal envelopes' seen in SEM micrographs (Figure 4-3). In all cases the crystal chambers are external to the plasmalemma. The crystal origin is most precisely described as *extraplasmic* because the epidermal cell wall, albeit very thin and immature at the time of crystal nucleation, is external to the COM crystals. This mode of extraplasmic nucleation has been observed in growing crystalliferous astrosclereids of *Nymphaea* (Kuo-Huang, 1992). However, in one TEM micrograph from that study, a crystal was seen floating in the cytoplasm. This observation was either an isolated occurrence and/or caused by the plane of section.

The development of a crystalliferous cuticle in *Chamaecyparis lawsoniana* involved "membranous crystal-initiating structures" that originated from "precipitation membranes" (Oladele, 1982). Cuticular transpiration creating a zone of high concentration of Ca^{2+} ions in the cell wall, and oxalic acid in the cytoplasm causing high concentration of oxalate ions near the plasmalemma were hypothesized to create spontaneous formation of the "precipitation membranes". These structures were depicted initiated some distance away from the plasmalemma (Oladele, 1982). Oladele's interpretation of periclinal growth of small peripheral crystals within the membranous compartment and subsequent coalescing with later-formed crystals to yield a

large, single crystal is difficult to visualize. For a single crystal to form, smaller crystals have to dissolve first and yield their constituent ions to the aqueous medium enclosed by the membranous compartment. Only then can these ions contribute to the growth of a larger, single crystal. Cuticular crystals in *D. sanderiana* show an increase in size until crystal growth has stopped, no fluctuations of crystal size are observed. If smaller crystals did indeed exist in the initial "precipitation membranes" in Oladele's study, then a conglomerate of small crystals would have been observed.

In coniferous gymnosperms the extracellular crystals originated *in situ* within the cell wall (Fink, 1991b). Most crystal growth in the mesophyll originated directly from the outermost layer of the cell wall. A different mode of extracellular crystal deposition was observed in *Dracaena marginata* and *Sempervivum wulfenii* (Fink, 1991a). In the former species, crystals originated from "deeper layers within the cell walls", and in the latter species, crystals were initially seen as "free-floating in the cytoplasm" and then secondarily attaching themselves to the internal cell walls. A detailed developmental sequence for either species was not a part of Fink's study.

A difference apparently exists in the mode of extracellular/extraplasmic crystal deposition between angiosperms and coniferous gymnosperms. This study and previous ones (Kuo-Huang, 1992; Berg, 1994) suggest that such crystallization in angiosperm species occurs in crystal chambers, which originate from the plasma membrane.

Growth of cuticular crystals in *D. sanderiana* involved RER-derived vesicles. These vesicles traverse the plasma membrane on their way to the apoplastic space and the developing crystals. Association of ER with

extracellular crystals has been reported in organic secreting trichomes of chickpea (*Cicer arietinum*), where a "tubular-vesicular membrane network opened into the hole that contained a calcium oxalate crystal" (Lazzaro and Thomson, 1989). Since the crystals were not the primary focus of Lazzaro and Thomson's study, very little information of that process was given.

Golgi-derived vesicles are known to be involved with calcification phenomenon in cocolithophorids (Borowitzka, 1989). In these algae the calcite CaCO_3 is deposited in the form of delicately-sculpted plates within special Golgi-derived vesicles. The involvement of RER-derived vesicles in the crystal deposition process in *D. sanderiana* is of substantial interest because it implies active participation of the living protoplast. The nature of transported material(s) at this point is unknown and may be Ca^{2+} and/or oxalate ions. It is of particular interest that the onset of crystal deposition occurs so early in leaf ontogeny and that large quantities of CO are precipitated. At this developmental stage the primordial cells are meristematic, and therefore, sinks for various substances necessary for cell growth and expansion. In addition, these cells are effectively sealed from any significant irradiation (due to the morphology of the monocot shoot apex) and therefore, are not photosynthetically-competent. All facts point to oxalate derivation from sources other than photosynthetic respiration (see Chapter 3, pathways of oxalate synthesis). The oxalate could be obtained symplastically from mature tissues and/or it could be synthesized locally from substances found in the primordial cells themselves.

Glycolic and glyoxylic acids have been implicated as oxalate sources for CO crystals in *Lemna minor* (Franceschi, 1987). Photorespiratory glycolate was

an unlikely oxalate source for CO crystals because they developed in very young tissues of *L. minor*. Under favorable conditions crystals could form within one hour and such rapid transport of glycolic acid from mature tissues is improbable (Franceschi, 1987). In addition, dark-grown, heterotrophic plants formed four times the number of crystal idioblasts compared to light-grown plants; thus, providing further support for a non-photorespiratory glycolate source. A likely source of oxalate is thought to be L-ascorbic acid which is considered a normal substance in higher plants (Loewus, 1980) and occurs in high levels in shoot tissues (Nanda and Tayal, 1976). Ascorbic acid is dependent on carbohydrate supply (Franke, 1959), and starch grains are present in developing leaf primordium of *D. sanderiana*. Arguably it could be hypothesized that the rough endoplasmic reticulum in *D. sanderiana* is actively involved in crystal precipitation by secretion and transport of oxalate and/or Ca^{2+} -filled vesicles and oxalate is derived from a carbohydrate supply in the form of starch.

The existence of COM crystals in association with the apoplast of mesophyll cells and intracellular spaces between the leaf mesophyll (Figure 4-15) indicates a system which processes large quantities of CO. Such a mode of crystal deposition is of widespread occurrence in the genus *Dracaena* and reminiscent of coniferous gymnosperms (see Chapter 7). Curiously, more crystals are found on the surfaces of achlorophyllous mesophyll cells of *D. sanderiana* than on the chlorophyllous ones. Some achlorophyllous cells in *D. sanderiana* have considerably more crystals associated with their walls than other achlorophyllous cells. Possibly, an explanation for this nonuniform crystal distribution is the location of the achlorophyllous cells with respect to

the internal tissues and more specifically, the vascular tissue. If cells originated in close proximity to the vascular tissue/xylem, more crystals would be found in their walls because Ca^{2+} moves within the transpiration stream and its concentration is naturally high near the xylem. Conversely, fewer crystals would be found in achlorophyllous cell walls distal to the vascular tissue due to the lower Ca^{2+} concentration in their apoplastic space. A layer of achlorophyllous cells is found adaxially and abaxially in subepidermal leaf areas, and is related to the chimera nature of the plant (Vladimirova, 1996). These cells are located some distance from the vascular tissue and have fewer crystals attached to their cell walls.

Borchert (1984) supported the view that chlorophyllous cells are likely to be more efficient in extruding Ca^{2+} ions compared to achlorophyllous ones due to the higher energy supply of ATP from photosynthesis. He found that the induction of achlorophyllous cells into crystal idioblasts occurred at lower Ca^{2+} concentrations ($<0.3\text{mM}$) than in chlorophyllous ones. CO crystals accumulated predominantly in the achlorophyllous cells of callus cultures consisting of both achlorophyllous and chlorophyllous cells (Horner and Franceschi, 1981). Their findings agree with the present study.

CHAPTER 5
INTRACELLULAR CALCIUM OXALATE DEPOSITS IN *DRACAENA SANDERIANA*

Calcium Oxalate Dihydrate Crystals

In addition to apoplastic deposits, *D. sanderiana* forms crystals in intracellular locations. When mature leaf mesophyll cells are isolated by maceration, variously-sized rod-like and prismatic crystals are evident (Figure 5-1). Their birefringence is lower and their morphology is apparently dissimilar to COM. The intracellular rod-like deposits are small (approx. 4-5 μ m) (Figure 5-1B), and some exhibit twinning (Figure 5-1C).

Prismatic crystals resembling cubes are most abundant in macerated immature leaf primordial cells (Figure 5-2) before the onset of cuticular COM deposition, and these vary from 2 to 4.5 μ m in length. These crystals are characterized by a four-fold axis of symmetry, and expression of (101), (011), ($\bar{1}$ 01), ($\bar{0}$ 11), (10 $\bar{1}$), (01 $\bar{1}$), ($\bar{1}$ 0 $\bar{1}$) and ($\bar{0}$ 1 $\bar{1}$) faces that are typical of the tetragonal-dipyramidal class (Figure 5-2B-D). The {101} form encloses a tetragonal pyramid at both ends of the crystals, and the (100), (010), ($\bar{1}$ 00), and ($\bar{0}$ 10) faces enclose the parallel sides of the tetragonal prism. The final crystal form is a combination of a tetragonal dipyramid and a tetragonal prism (Figure 5-2E). Rotational twins (Figure 5-2D) are common (Figure 5-2E).

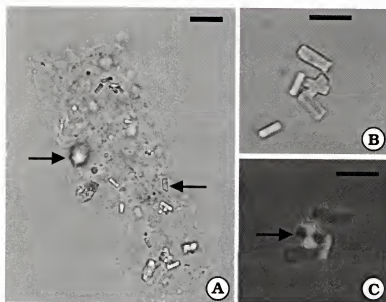


Figure 5-1. LM micrographs of calcium oxalate dihydrate (COD) crystals isolated from the mesophyll of *Dracaena sanderiana*.

A. Achlorophyllous mesophyll cell with numerous intracellular rod-like crystals (left arrow) and a single prismatic crystal, which is not in the same focal plane (right arrow). Bar = 10 μ m.

B. Isolated rod-like crystals. Bar = 5 μ m.

C. Identical to B but viewed in polarized light with crossed polars. Dark rod-like crystals are in extinct position and so is one of the crystals forming a cross, which indicates twinning (arrow). Bar = 5 μ m.

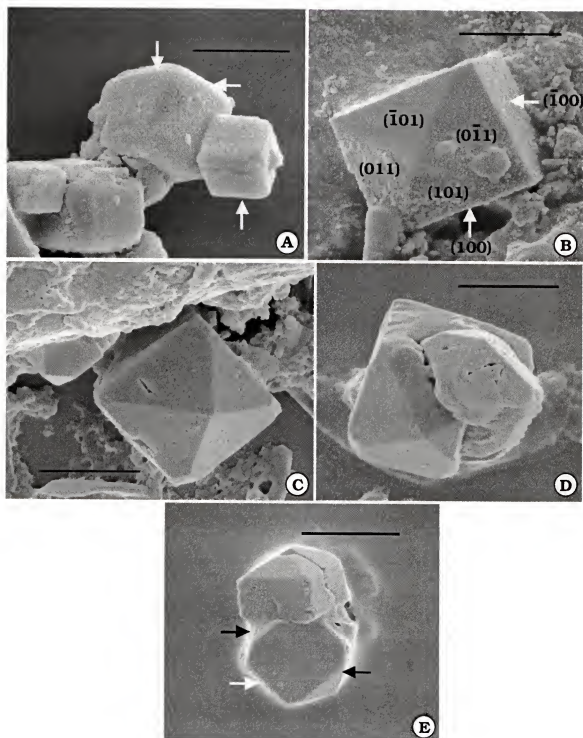
Figure 5-2. SEM micrographs of intracellular crystals with typical COD morphology isolated from immature leaves of *Dracaena sanderiana*.

A. Crystals completely enclosed in an envelope of amorphous material (arrows). Bar = 1.5 μ m.

B and C. COD Crystals display unobstructed faces and a morphology typical of the tetragonal system: one four-fold axis of symmetry resulting in tetragonal pyramids at both crystal ends. The faces typical of the tetragonal-dipyramidal class are expressed. The faces of the pyramid located at the opposite end of the crystal are not visible, their indices are $(10\bar{1})$, $(0\bar{1}1)$, $(\bar{1}01)$, and $(01\bar{1})$. Bars = 1.7 and 1.5 μ m, respectively.

D. A twinned crystal, likely a growth twin. Bar = 2 μ m.

E. A twinned crystal. Note that the shape of the COD crystals is a combination of a tetragonal dipyramid (black arrows) and a tetragonal prism (white arrow). Bar = 2 μ m.



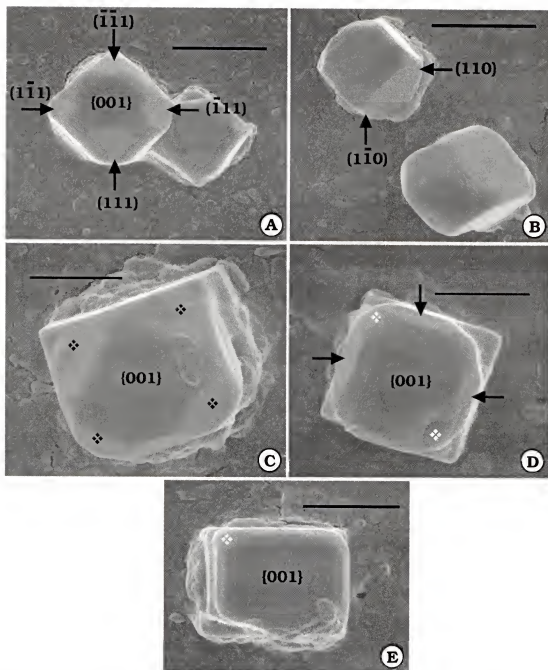


Figure 5-3. SEM micrographs of intracellular crystals with atypical COD morphology isolated from immature leaves of *Dracaena sanderiana*.

A-E. Crystals display {111} faces (black stars in C) and {110} faces (B) which are not consistent with the typical dipyrnmidal COD morphology (compare with Figure 5-2). The pinacoid {001} is present.

D-E. {101} faces (arrows) are small compared to the typical COD morphology (compare with Figure 5-2). Crystals exhibiting some rounded corners (white stars).

Bars = 4.3, 3.8, 2.3, 3 and 2.7 μ m, respectively.

Occasionally the intracellular crystals are isolated with an amorphous envelope around them (Figure 5-2A), but most deposits have faces lacking any foreign material (Figure 5-2B-E). Some crystals isolated from mature leaves have $\{111\}$ and $\{110\}$ crystal faces that differ from the the typical COD crystals (Figure 5-3A-E) including the pinacoid $\{001\}$ is apparent (Figure 5-3C). Some rounded crystal corners also are evident (Figure 5-3D-E). Both crystal morphology and X-ray diffraction data (Table 5-1) confirm that the intracellular crystals are COD. None of the preparations from macerated leaf primordial cells contain any rod-like shaped crystalline substances that could be observed with a scanning electron microscopy, although X-ray data indicate all isolated intracellular deposits, excluding raphides, are COD.

Internally immature leaf primordial cells feature vacuoles with pronounced angular outlines (Figure 5-4A-D) that are likely associated with the intracellular COD crystals. These vacuoles appear connected to flat sheets of rough ER. A single RER profile extends from one cell pole, produces a crystal vacuole in the cell's center, and traverses circuitously through the intervening cytoplasm and connects with RER structures at the opposite cell pole (Figure 5-4D). Paramural bodies also are present in the immature mesophyll cells (Figure 5-4C).

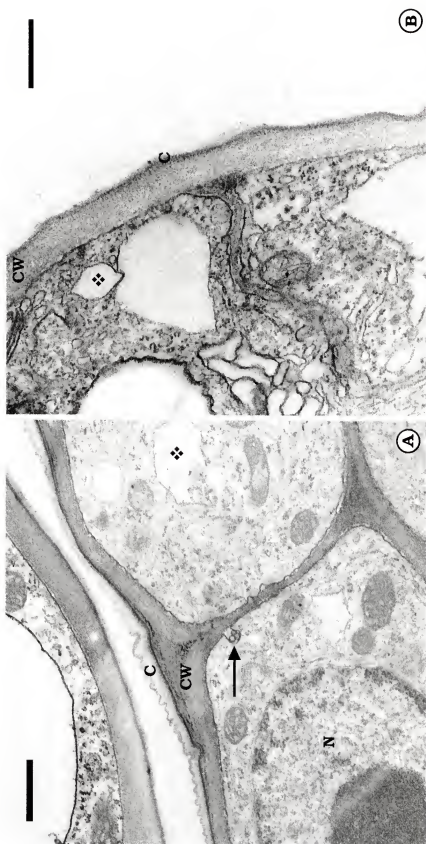


Figure 5-4. TEM micrographs of internal crystal structures in immature epidermal and mesophyll cells in *Dracaena sandertiana* leaf primordium.

A and B. Note angular outlines of crystal vacuoles (stars). Paramural bodies in association with the radial epidermal cell wall (arrow). Bars = 0.7 and 0.5 μ m, respectively.

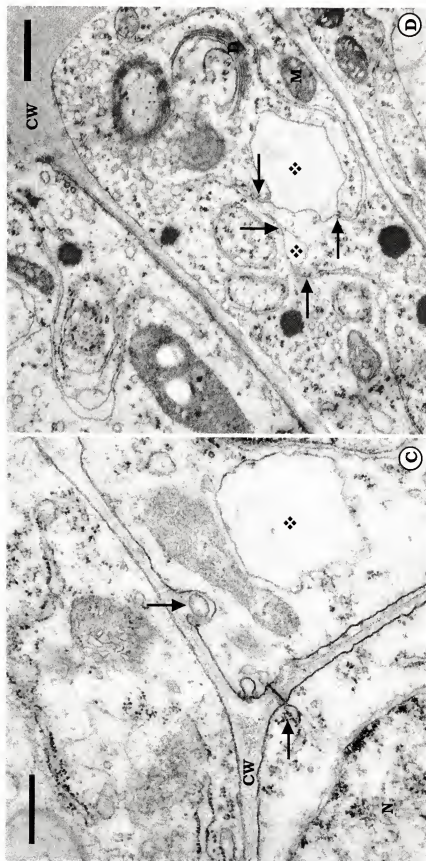


Figure 5-4 continued.

C. Note rectangular-shaped crystal vacuole (stars). Two paramural bodies are present adjacent to the cell wall (arrows).

D. Crystal vacuole (stars) membranes are continuous with two profiles of ER (arrows). Bars = 0.5 and 0.7 μ m, respectively.

Table 5-1. Comparison of ASTM data of calcium oxalate dihydrate with intracellular crystals extracted from the mesophyll of *Dracaena sanderiana*.

ASTM weddellite $\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}^z$		<i>Dracaena sanderiana</i>	
D, Å ^y	I/I ₀ ^x	D, Å	I/I ₀
*6.18 ^w	100	6.21	100
*4.42	30	5.99	25
*3.78	65	3.72	16.5
2.41	16	2.41	20.3
2.24	25	2.25	13.9
1.90	16	-	-

^zASTM data were obtained from Joint Committee on Powder Diffraction Standards (JCPDS) – International Centre for Diffraction Data, 1996.

^yD is the wavelength spacings in Ångstroms.

^xI/I₀ is relative intensity of diffraction response for each analysis.

^wThe three major peaks are indicated by an asterisk (*) in each analysis.

Calcium Oxalate Monohydrate Raphides

As indicated in Chapter 4, *D. sanderiana* leaf primordia develop intracellular crystal idioblasts which contain bundles of numerous individual acicular crystals termed raphides (Figure 5-5A-D). High birefringence, typical crystal morphology and X-ray diffraction data (Table 5-2) confirm that the raphides are composed of COM. The length of mature raphides can reach 80-90µm (Figure 5-6A) and they have sharp, pointed ends and irregular edges (Figure 5-5D). The raphide bundle is located in a central position in the crystal idioblast (Figure 5-6A), and the vacuole, which contains a raphide bundle can occupy the entire volume of the crystal idioblast (Figure 5-6D). One hundred to

one-hundred and fifty individual crystals can be assembled in one bundle (Figure 5-6D). As raphides grow they occupy a larger volume of the cell and the crystal idioblast increases in diameter (in transverse section) (Figure 5-6B-D). Their morphology changes from rectangular (Figure 5-6C) to hexagonal with pointed ends (Figure 5-6D; Figure 5-7A-B) in transverse section. In their final shape raphides appear flattened, approximately 1.5 μ m wide and 0.8 μ m high (Figure 5-7C).

Calcium oxalate monohydrate raphides appear earlier in the leaf primordial ontogeny when compared to cuticular COM crystals (Figure 5-5B; Figure 5-6E). Most raphide bundles have reached their mature size at the time when cuticular crystal deposition has just been initiated. Raphide growth does not proceed simultaneously for all bundles at similar developmental stages (Figure 5-6E). In a mature leaf all raphide idioblasts are oriented with their long axis parallel to the long axis of the leaf. The orientation of the long crystal axis is more random during their growth stage (Figure 5-6E).

Ultrastructurally the raphide idioblasts exhibit several distinctive characteristics. Paracrystalline bodies with closely-spaced subunits are observed (Figure 5-7). They are located randomly in the cell vacuole and measure approximately 1 μ m in transverse section. All raphides are embedded in a mucilagenous matrix (Figure 5-8A). In a developing leaf primordium individual raphides are oriented randomly with respect to each other, and large spaces exist between individual crystals.

Figure 5-5. LM and SEM micrographs of raphide idioblasts and isolated crystals in immature and mature *Dracaena sanderiana* leaves.

A-B. Photographs taken with polarized light and crossed polars.

A. Two isolated individual raphide crystals recognizable by their acicular morphology. The lower crystal is oriented such so that maximum brightness is observed; the top crystal is in partial extinct position. Extraplasmic cuticular COM crystals also are present. Their birefringence also is a function of the orientation. Bar = 10 μ m.

B. Growing raphide bundles in the base of a leaf primordium. Note high birefringence typical of COM. Bar = 50 μ m.

C. Isolated single raphide. Bar = 23 μ m.

D. Note irregular outlines of the crystal edges (arrow). Bar = 1.5 μ m.

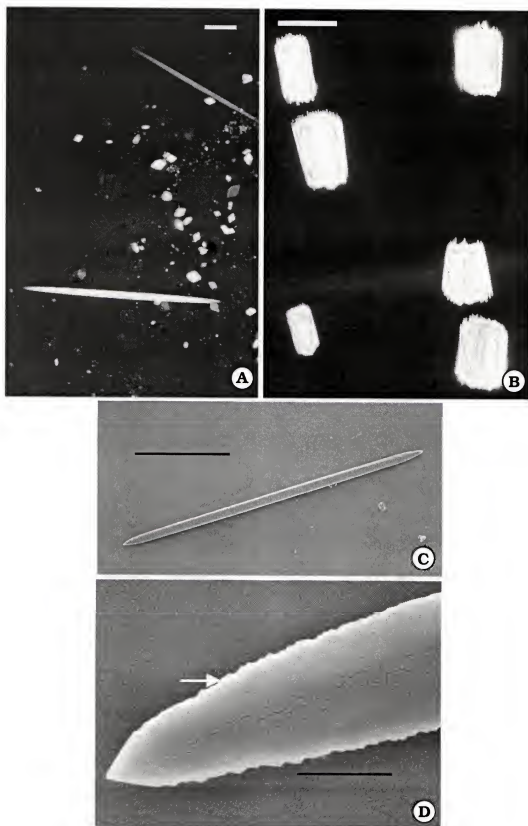


Figure 5-6. LM micrographs of raphide idioblasts in immature *Dracaena sanderiana* leaves.

A. A single raphide idioblast isolated from macerated leaf tissue. Note size of the vacuole containing raphide bundle with respect to the cell. Bar = 50 μ m.

B-D. Transverse sections of progressively older cells along the length of a leaf primordium. Arrows, developing raphide bundles. The shape of the individual crystals changes from rectangular (C) to hexagonal (D) in transverse section. Bars = 3.8, 2.3, and 3 μ m, respectively.

E. Viewed in polarized light with crossed polars. Developing raphide bundles at the base of the leaf primordium. The primordium tip is toward the top. Note that long axis of the raphides in the largest bundle is parallel to the long axis of the primordium, but the long axes of the other two smaller bundles are not. Bar = 50 μ m.

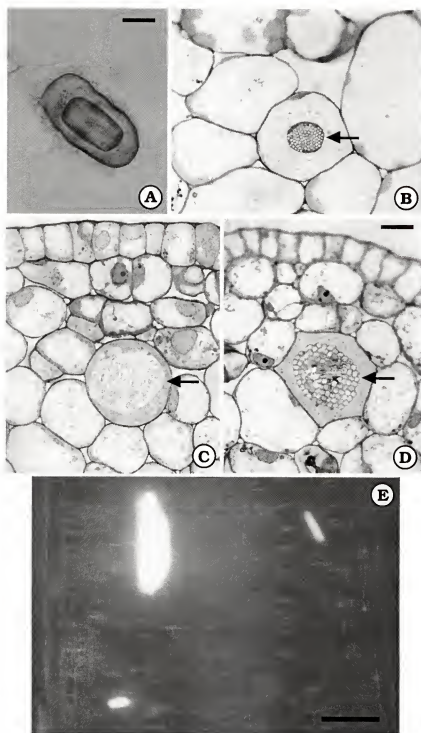


Table 5-2. Comparison of ASTM data of calcium oxalate monohydrate with intracellular raphides extracted from the mesophyll of *Dracaena sanderiana*.

ASTM whewellite $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}^*$		<i>Dracaena sanderiana</i>	
D, Å ^y	I/I ₀ ^x	D, Å	I/I ₀
*5.93 ^w	100	5.93	100
5.79	30	5.79	25
*3.65	70	3.66	54.8
3.01	10	-	-
*2.97	45	2.88	43
2.92	10	-	-
2.84	10	-	-
2.49	18	2.49	10
2.36	30	2.32	12.8
2.08	14	-	-
1.98	10	1.96	8.4
1.85	6	1.84	2.8
1.82	6	1.82	2.8

*ASTM data were obtained from Joint Committee on Powder Diffraction Standards (JCPDS) – International Centre for Diffraction Data, 1996.

^yD is the wavelength spacings in Ångstroms.

^xI/I₀ is relative intensity of diffraction response for each analysis.

^wThe three major peaks are indicated by an asterisk (*) in each analysis.

In a mature leaf spacing between the individual crystals in the bundle is decreased, and their orientation is regular and similar to hexagonal closest packing (Figure 5-8B). Individual mature raphides are surrounded by crystal chamber ($\approx 4\text{nm}$ -thick) which appears as a thick line around every raphide (Figure 5-8B).

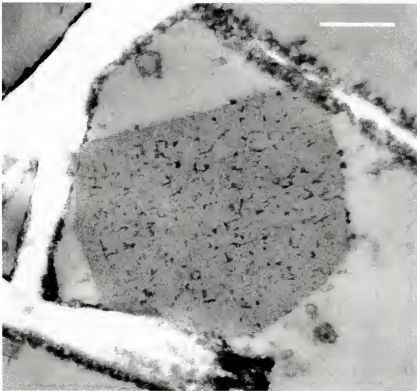


Figure 5-7. TEM of a raphide paracrystalline body with closely spaced subunits. The white areas are empty spaces left by raphides. Bar = 0.3 μ m.

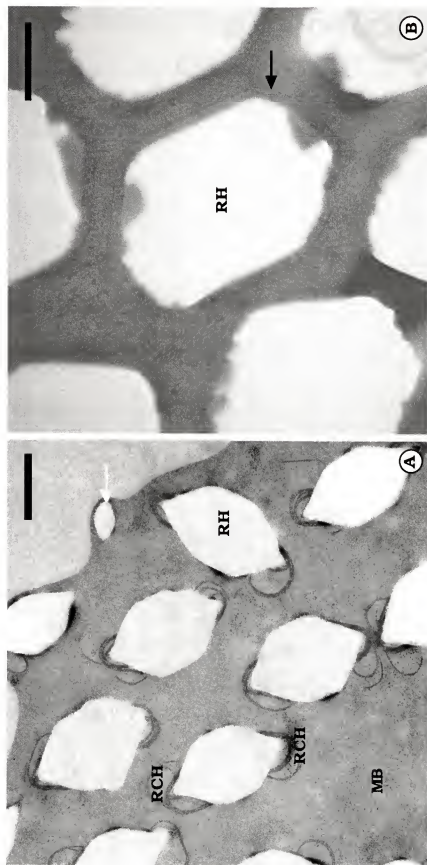


Figure 5-8. TEM micrographs of raphide idioblasts in immature and mature *Dracaena sandertiana* leaves.

A. Raphide bundle in a developing leaf primordia. Note the larger spaces between the individual raphides compared to B, and the irregular orientation of the crystals with respect to each other. White arrow, developing raphide. White holes are left by crystals, which are not impregnated by resin, and therefore fall out during sectioning. Bar = 0.7 μm .

B. Raphide bundle in a mature leaf. Note the hexagonal outlines and the crystal chamber (arrow). Bar = 0.3 μm .

Figure abbreviations: RCH, raphide chamber; RH, raphide hole; MB, mucilaginous body, matrix.

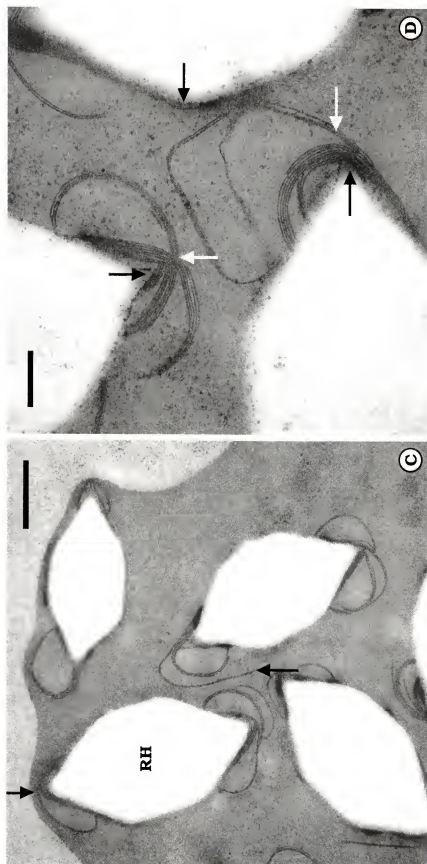
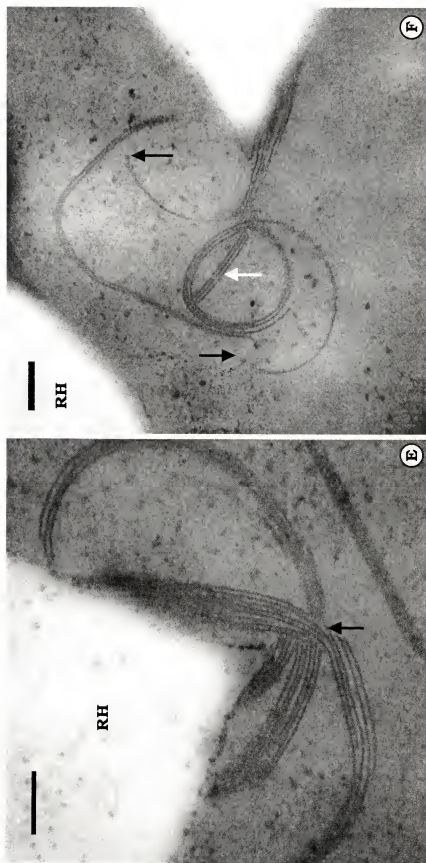


Figure 5-8 continued.

C. Note that the loop-like crystal chamber extensions are not connected with each other and do not extend to the edge of the mucilaginous body (down arrow). Note also the blind end of the crystal chamber extension (up arrow). Bar = 0.5 μ m.

D. The edges of the chamber walls are visible (right and down black arrows) and some of the side walls (left arrow). Some crystal chamber extensions are symmetrical (up white arrow), while other are asymmetrical (left white arrow). Bar = 0.2 μ m.



The most striking feature of *D. sanderiana* raphide bundles are the crystal chambers (Figure 5-8C-F). Each crystal is surrounded by a lamellate crystal chamber that is not connected to neighboring chambers or the edge of the mucilagenous matrix of the raphide bundle (Figure 5-8C). The chamber wall consists of a double membrane (Figure 5-8D). The chambers possess loop-like lamellate extensions along their wide ends (in transverse section), which may or may not be symmetrically situated along the chamber walls (Figure 5-8D). The length of the crystal chamber extensions ranges from 0.5 μ m to 1.6 μ m. Some extensions end blindly without completing a full loop (Figure 5-8C). The plane of sectioning may have caused the blind ends of some loops.

The lamellate extensions are connected to the sharp edges of the chamber walls where they cross each other (Figure 5-8E). The loop-like extensions are connected to only one side of the chamber wall in some instances (Figure 5-8F). Some lamellate parts of the loop-like extensions are not connected to other lamellate parts of the same extension.

Discussion

Calcium Oxalate Dihydrate Crystals

This study conclusively demonstrates the existence of two hydrate forms of CO, COM and COD, in the same plant species. Reported occurrences of COD crystals in plants are scarce, and conclusive reports of their chemical identity are even fewer. In most instances, crystal morphology has been the only definitive feature that could be used in analysis. The following is a list of confirmed reports on intracellular COD in plants (determined by X-ray diffraction).

- Solanaceae:
 - *Capsicum annum* – solitary prisms (Wagner, 1983) and druses (Horner and Wagner, 1992);
- Begoniaceae:
 - *Begonia* sp. - solitary prisms (Horner and Zindler-Frank, 1981);
 - *B. maculata*, *B. manicata*, *B. metallica* - solitary prisms and druses (Al-Rais et al., 1971);
- Labiatae:
 - *Coleus* sp. – solitary prisms and druses (Al-Rais et al., 1971);
 - *Beta vulgaris* – solitary prisms and cylindrics (Al-Rais et al., 1971);
- Cactaceae:
 - *Echinomastus intertextus*, *Echinocactus horizonthalonius*, *Escobaria tuberculosa* - druses (Rivera, 1973);

The following is a list of some unconfirmed reports on intracellular COD in plants (crystal morphology is the only criterion).

- *Telfairia* sp. - solitary prisms (Okoli and McEuen, 1986);
- *Acacia senegal* - solitary prisms (Parameswaran and Schultze, 1973);
- *Aglaonema modestum*, *Hydrosome rivieri* – solitary prisms (Genua and Hillson, 1985).

Horner and Lersten (1999) reported extracellular eruptions from papillae in leaf trichomes of *Cornus mas* which bore resemblance to COD morphology. In contrast to higher plants, the majority of CO extracellular deposits in fungi are COD (Whitney, 1989), and some deposits display a remarkable diversity of shapes and forms (Arnott, 1995) that are not readily reconcilable with the morphology of synthetic COD.

Intracellular COD crystals in *D. sanderiana* display morphology typical of the tetragonal-dipyramidal class, with expression of $\{101\}$ faces enclosing two tetragonal pyramids at both crystal ends. However, development of some unexpected faces was also present, with these faces being $\{100\}$ faces that enclose the tetragonal prism. This crystal form (combination of a tetragonal dipyramid and a tetragonal prism) has been documented in *Begonia* (Horner and Zindler-Frank, 1981) and *Capsicum* (Wagner, 1983; Horner and Wagner, 1992). Previously identification of crystal faces has not been attempted. Frey-Wyssling (1981) illustrated twinned COD crystals which appear similar to the crosses observed with light microscopy in *D. sanderiana* (Figure 5-1). The unusual crystal morphologies in the COD crystals in *D. sanderiana* resulting from expression of $\{001\}$, $\{111\}$, and $\{110\}$ faces have not been documented previously.

Figure 5-9 illustrates the changes in crystal morphology produced by the development of additional faces of COD. The rod-like crystals observed with light microscopy (Figure 5-1) could be easily explained by the relative expression of $\{100\}$ faces compared to the typical $\{101\}$ faces. Crystal habit modifications of COD have been achieved by growth of crystals in presence of α,ω -dicarboxylic acids (Stevens et al., 1999). Suberic acid, in particular, was shown to be specific for COD by creating a structural motif which mimicked the $\{110\}$ face and generated an unusual habit with expression of these high energy planes.

Crystals grown in presence of various macromolecules, specifically acidic proteins, have been known to exhibit modified crystal habit (Addadi and

Weiner, 1989; Gower and Tirrell, 1998). Development of these unstable crystal faces occurs due to interactions with constituents of the solution (i.e., acidic macromolecules).

Intracellular COD in *D. sanderiana* show lower monocrystalline/twin ratio compared to cuticular COM (see Chapter 4). Cody and Horner (1984) experimentally showed that *in vitro* twinning of COM occurred during conditions of supersaturation of the solution at the initial time of crystal formation. The degree of supersaturation may be attributed to the genetically-controlled rate at which the plant produced and combined the constituent ions necessary for crystal precipitation (Cody and Horner, 1983).

Space delineation is one of the most distinctive features of biologically controlled biomineralization (Lowenstam and Weiner, 1989). Lipid bilayers are the most common way of sealing off a pre-determined compartment for biomineralization purposes. This sealing off process allows selective uptake of ions and provides a means to control the concentration and composition of the initial solution from which the mineral forms (Lowenstam and Weiner, 1989). The extent of participation of the vesicle walls in the precipitation process is unknown. Although mineralization within lipid bilayer-bound vesicles is very common, the vesicle membranes may not be actively involved in the biomineralization process. One well-documented example in which the vesicle walls are directly involved in nucleation of hydroxyapatite are the extracellular "matrix vesicles" in mineralized tissues of vertebrate organisms (Lowenstam and Weiner, 1989).

Intracellular COD crystals in *D. sanderiana* developed in vesicles directly derived from RER. The crystal vacuoles curiously remain attached to the parent

RER membranes even after the crystals have reached final size (Figure 5-4A-D). The mechanism of uptake and/or transport of ions through and/or along the vesicle walls in order to achieve supersaturation of the solution from which COD is precipitated is presently unknown in this study. Once the mineralization process is initiated water molecules have to be removed or dissolution will occur. How this process occurs is unknown. COD precipitation inside *D. sanderiana* cells, however, is controlled by the elaboration of RER crystal vacuoles and crystal morphology is modified by the development of unstable crystal faces.

Calcium Oxalate Monohydrate Raphides

The raphides in *D. sanderiana* are composed of COM, which is consistent (with one exception) with other reports in the literature. Raphide-containing cells in *D. sanderiana* exhibited characteristics typical of System II crystal idioblasts as defined by Horner and Wagner (1995) (see Chapter 2). This system is exemplified by the monocotyledonous raphide idioblasts in *Typha*, *Vanilla* and *Yucca*, and is typified by lamellate sheaths around the chamber walls, mucilage-like material surrounding the developing crystal chambers and paracrystalline bodies with closely spaced subunits (Horner and Whitmoyer, 1972; Wattendorff, 1976; Tilton and Horner, 1980). The loop-like extensions of the crystal chambers in *D. sanderiana* are very similar to the chamber wall extensions of *Agave* raphides (Wattendorff, 1976). However, in *Agave*, these structures were larger, displayed multiple lamellae and formed closed loops, while in *D. sanderiana* some of the raphide chamber extensions were single lamellae and either terminated blindly in the surrounding mucilagenous matrix

or were less symmetrically-oriented than the loop-like extensions in *Agave* raphide chambers.

Unlike the crystal lamellae in *Typha*, which were continuous with lamellae from neighboring crystals (Horner et al., 1981), the chamber lamellae in *D. sanderiana* did not anastomose with other crystal chambers and did not show any continuity with the vacuolar membrane (tonoplast). The paracrystalline body (Figure 5-7) is an enigmatic structure and no satisfactory explanation of its nature and function are known. The ordered substructure (i.e., paracrystalline body) is not observed frequently (Barnabas and Arnott, 1990).

During their growth *D. sanderiana* raphides change from four-sided to six-sided in transverse section. Wattendorf (1976) provided a growth sequence in monocotyledonous raphides that could be applied to *D. sanderiana* raphides (Figure 5-10), and is similar to System II crystal growth (see Figure 2-3). Raphides in *D. sanderiana* are similar morphologically to raphides in some monocots, namely: *Typha* (Horner et al., 1981), *Agave* (Wattendorff, 1976), and *Ornithogalum* (Tilton and Horner, 1980) in having at least 6 sides, rather than the typical 4 sides observed for many other monocots and dicots (Wattendorff, 1976).

Raphides represent the most intriguing shape of COM in plants because this crystal type has not been reproduced synthetically. Theories concerning the development of these crystal morphologies are numerous, and some theories have implicated macromolecules (i.e., proteins and complex polysaccharides) (Webb et al., 1995; Webb, 1999). Crystal chambers may act as molds and control both the shape and size of the crystals within them (Arnott

and Pautard, 1970). An antigen associated with crystal idioblasts in the stomium and connective tissue of the anthers of *Nicotiana tabacum* has been found (Trull et al., 1991). This antigen was a protein and was not tightly membrane-bound but was closely localized around the crystals.

A wide variety of additives have been shown to alter COM morphology and produce crystals that resemble certain crystal habits found in plants (Cody and Horner, 1984; Cody and Cody, 1987; Stevens et al, 1999). One hypothesis of how macromolecules (acidic proteins) affect the mineral phase is the so-called polymer-induced liquid precursor (PILP) process (Gower and Tirrell, 1998). Non-equilibrium crystal morphologies were generated in a solution crystallization of calcium carbonates in the presence of polyaspartic acid. The strongly acidic polypeptide induced the formation of an aqueous biphasic, and droplets phase-separated accumulated as a precursor to the mineral phase (Gower and Tirrell, 1998). The final mineral retains the shape of the precursor, and in essence, is "molded" to form unusual morphology. Similar phase separation and formation of a liquid precursor has been shown in experiments with calcium oxalate (Malpass and Gower, 1999). Whether such a phenomenon occurs in plants is presently unknown.

Why COM is the predominant hydration form in raphides is unknown. Also the factors, which determine the hydration state of other known CO crystals are unknown. The two hydration forms of CO originate under different chemical conditions (Frey-Wyssling, 1981) and may occur at different developmental stages (Horner and Wagner, 1980). The hydration form of CO crystals may affect the plants' ability to dissolve the crystal and utilize the constituent ions. This finding is particularly interesting in the light of the fact

that COD crystals in *D. sanderiana* are most abundant in meristematic primordial leaf cells before the onset of cuticular COM deposition. As cuticular crystals are formed COD crystals decrease in quantity evidenced by the lower quantity of COD crystals in mature leaf mesophyll cells. The COD crystals possibly are being used as a temporary storage form of Ca^{2+} and/or oxalate, and later are dissolved and incorporated into the cuticular COM crystals and/or the intracellular COM raphides.

What determines that cuticular crystals and raphides in *D. sanderiana* are COM and the intracellular prisms are COD is unknown, but the constancy of CO forms in tissue-specific locations could possibly be the ultimate evidence of a highly developed phytosystem for biologically-controlled bimineralization. The type of mineral formed could conceivably reflect the molecular structure of the nucleation site (Lowenstam and Weiner, 1989). It has been proposed that the structure of the nucleation site is responsible for organisms forming aragonite and not calcite (both CaCO_3 polymorphs) (Hare, 1963).

Figure 5-9. Schematic illustration showing hypothetical growth modifications of crystal faces in the intracellular COD crystals.

A. Typical tetragonal COD bipyramids grown in vitro with no additives.

B. Development of tetragonal prisms $\{100\}$ in intracellular COD crystals in *Dracaena sanderiana* (compare with Figure 5-2B-E).

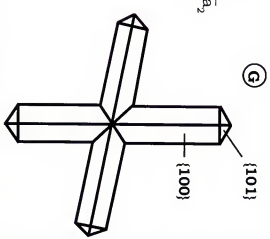
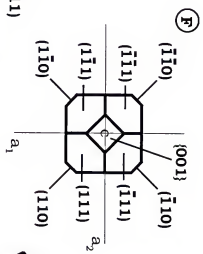
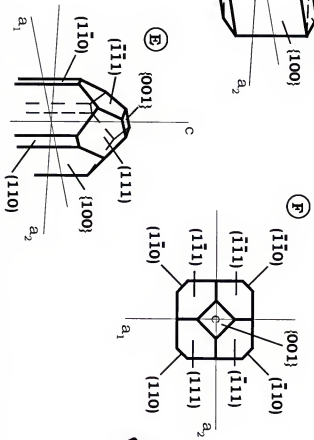
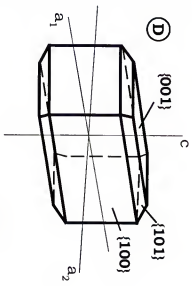
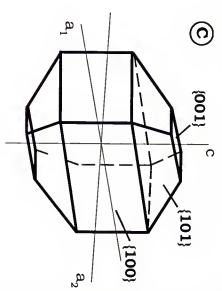
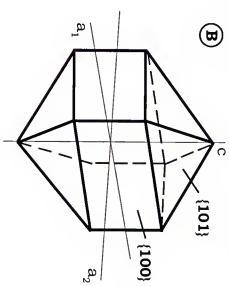
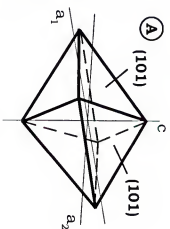
C. Development of $\{001\}$ pinacoids in intracellular COD crystals in *Dracaena sanderiana*.

D. Growth of the $\{001\}$ pinacoids is inhibited resulting in large $\{001\}$ and very small $\{101\}$ faces (compare with Figure 5-3D-E).

E. Additional habit modifications with development of $\{111\}$ and $\{110\}$ faces (compare with Figure 5-3A-C).

F. Same as E seen down the c-axis.

G. Twinning of COD crystals resulting in appearance of 'crosses' (compare with Figure 5-1B-C). Note the large area of the $\{100\}$ faces compared to $\{101\}$ faces. Similar COD morphology has been observed in *Allium cepa* (Frey-Wyssling, 1981).



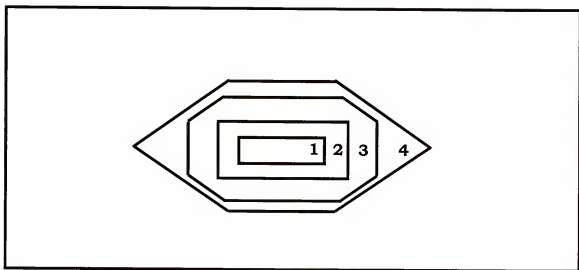


Figure 5-10. Schematic illustration showing a hypothetical growth sequence of the intracellular raphide COM crystals (adopted from Wattendorff, 1979).

1 and 2 represent earliest stages in crystal growth when raphides are rectangular in transverse section (compare with Figure 5-6C).

3 represents a later growth stage when raphides are octagonal in transverse section.

4 represents mature raphide morphology typified by a hexagonal shape (compare with Figure 5-8).

CHAPTER 6
EFFECT OF EXOGENOUS CALCIUM SUPPLY ON CRYSTALS IN *DRACAENA*
SANDERIANA

Results

Dracaena sanderiana plants respond to exogenous Ca^{2+} levels in remarkably different ways depending on whether they had been deprived of endogenous Ca^{2+} (mineral-deficient) or not (non-deficient) (Tables 6-1 and 6-2). The most striking feature of mineral-deficient plants grown in 0mM Ca^{2+} is that leaf primordia are devoid of intracellular raphide idioblasts (Figure 6-2B-F). Figure 6-1 illustrates two zones of maturation with respect to the elongating leaf primordium, namely: the raphide idioblast zone and the extraplasmic crystal zone. The extraplasmic crystal maturation zone occurs physiologically and anatomically later in mineral-deficient plants grown under 0mM Ca^{2+} compared to mineral-deficient plants grown in 3 and 7mM Ca^{2+} (Table 6-1). Very minute crystals ($<1\mu\text{m}$) are observed in the extraplasmic crystal maturation zone of mineral-deficient plants grown in 0mM Ca^{2+} (Figure 6-2G-H). These crystals remain rhombohedral (4-sided) throughout the crystal growth phase (Figure 6-2I-J), and final crystal size does not exceed $3\mu\text{m}$ (Table 6-1). Total crystal number per cell does not differ drastically from total crystal number per cell in plants grown under the other two Ca^{2+} regimes (Table 6-1).

Major differences are detected between mineral-deficient plants grown in 3 and 7mM Ca^{2+} . Plants grown in 7mM Ca^{2+} develop some extraplasmic crystals, which reach $10\mu\text{m}$ in length (Table 6-1 and Figure 6-4G-H).

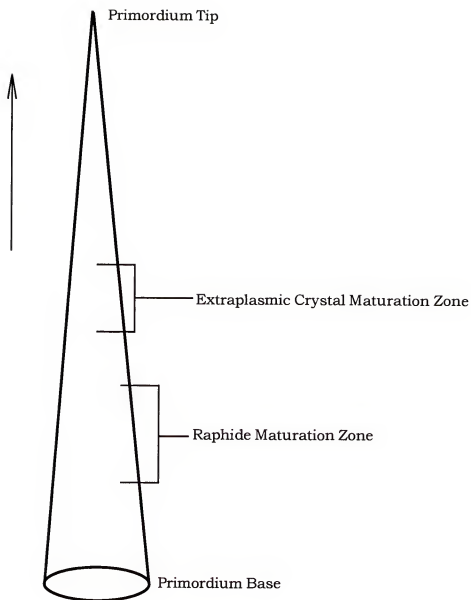


Figure 6-1. Schematic illustration of *Dracaena sanderiana* leaf primordium and zones of maturation of the intracellular raphide idioblasts and the extraplasmic cuticular crystals. Arrow indicates direction of leaf elongation.

Table 6-1. Cuticular- and raphide-related characteristics of leaf primordia from mineral-deficient *Dracaena sandariana* plants grown in three exogenous levels of Ca^{2+} .

Treatment	Crystal Maturation Zone (μm from primordium base)	Crystal Shape	Crystal Size	Crystal Number (per epidermal cell)			Number of Raphide Idioblasts
				<1-2 μm	3-5 μm	6-10 μm	
				Total*			
0mM Ca^{2+}	2940 ^z	Rhombohedral ^y	<1-3 μm	47 ^x	9	0	0
						56 ^{ns}	
3mM Ca^{2+}	2060	Rhombohedral to 5- or 6-sided	<1-8 μm	27	14	11	9
						52 ^{ns}	45
7mM Ca^{2+}	1720	Rhombohedral to 5- or 6-sided	<1-10 μm	31	10	15	11
						56 ^{ns}	54

^z Values are means of three replicates. Measurements were taken on primordia between 10-14mm in length.

^y Rhombohedral (diamond-shaped) with four sides as seen in surface view.

^x Values are means (rounded to integers) of three primordia, five epidermal cells per primordium.

^w Raphide numbers are counted in primordia with the specified lengths.

^v Values are rounded to an integer.

* Total crystal number per epidermal cell (the sum of all size crystals).

** Arrows indicate values subjected to statistical comparisons among crystal size groups and total crystal numbers per epidermal cell between Ca^{2+} treatments.

^{ns/ s} Means not significant/ significant at the 0.05 level.

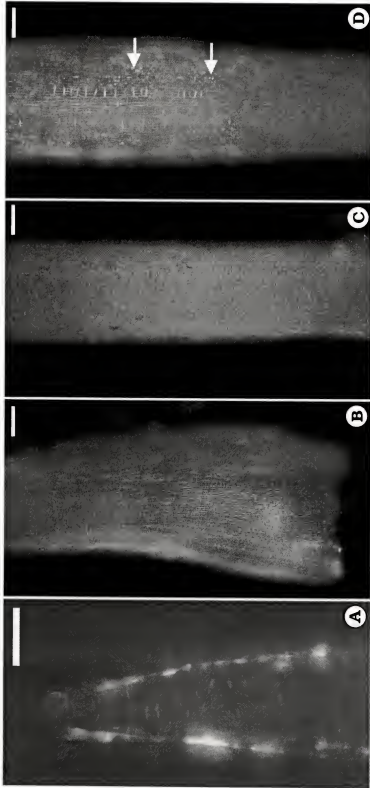


Figure 6-2. LM micrographs of leaf primordia from mineral-deficient *Dracaena sandertiana* plants grown in 0mM Ca^{2+} .

A-J. Viewed in polarized light with crossed polars.

A. Primordium length = 1mm. Note absence of any crystalline substances. The bright birefringence on the surface is residual and not related to the crystals under investigation. Bar = 50 μm .

B-D. Primordium length = 7mm. Basal (B), middle (C), and subterminal (D) portions of the primordium. Note absence of raphide idioblasts and weak birefringence of the cuticular crystals (arrows) in D. Bars = 100 μm .

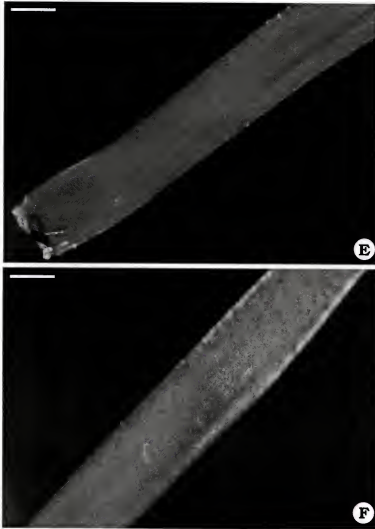


Figure 6-2 continued.

E-F. Primordium length = 12mm. Basal (E) and middle (C) portions of the primordium. Note absence of raphide idioblasts. Bar = 500 μ m.

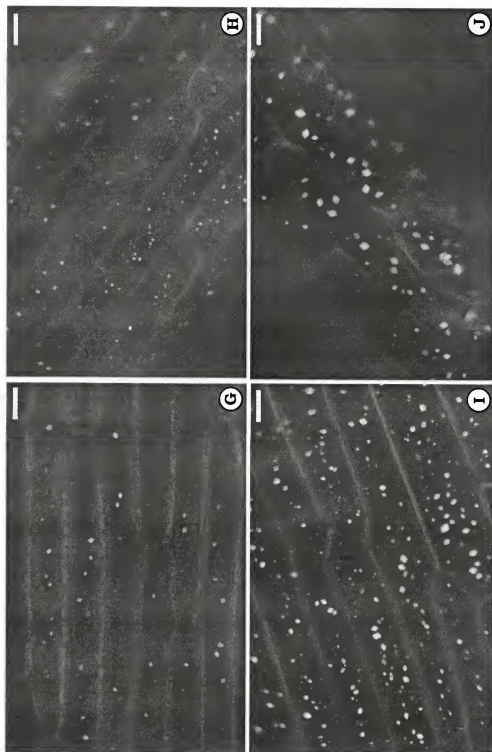


Figure 6-2 continued.

G-H. Primordium length = 5mm. Note rhombohedral shape and small size of developing cuticular crystals.

I-J. Primordium length = 7mm. Note final crystal size and shape (rhombohedral). Bars = 10 μ m.

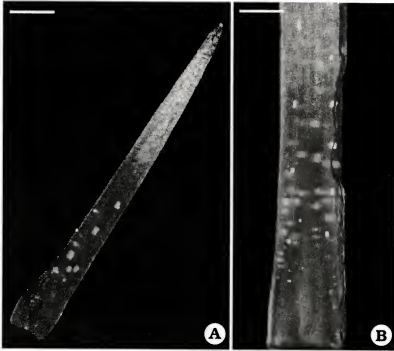


Figure 6-3. LM micrographs of leaf primordia from mineral-deficient *Dracaena sanderiana* plants grown in 3mM Ca^{2+} .

A-D. Viewed in polarized light with crossed polars.

A. Primordium length = 4mm. Note presence of raphide idioblasts. Bar = 500 μm .

B. Primordium length = 10mm. Basal portion of the primordium. Note abundance of raphide idioblasts. Bar = 500 μm .

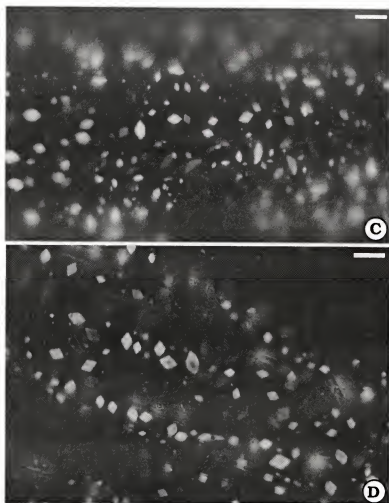


Figure 6-3 continued.

C-D. Primordia lengths = 4 and 10mm, respectively. Note shape and size of developing crystals (C), and final size and shape of cuticular crystals (D). Bars = 10 μ m.

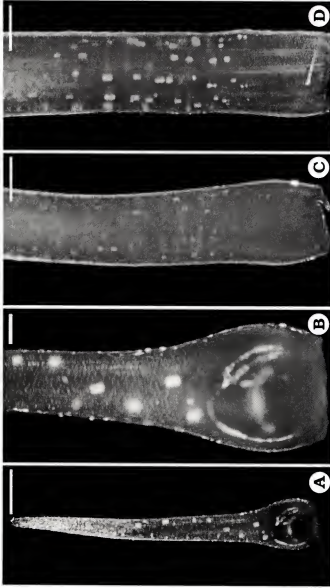


Figure 6-4. LM micrographs of leaf primordia from mineral-deficient *Dracaena sandieriana* plants grown in 7mM Ca^{2+} .

A-H. Viewed in polarized light with crossed polars.

A-B. Primordium length = 3mm. Note abundance of raphide idioblasts and the precocious development of cuticular crystals. Bars = 500 and 100 μm , respectively.

C. Primordium length = 10mm. Basal portion of the primordium. Note abundance of raphide idioblasts. Bar = 500 μm .

D. Primordium length = 13mm. Basal portion of the primordium. Note abundance of raphide idioblasts. Bar = 500 μm .

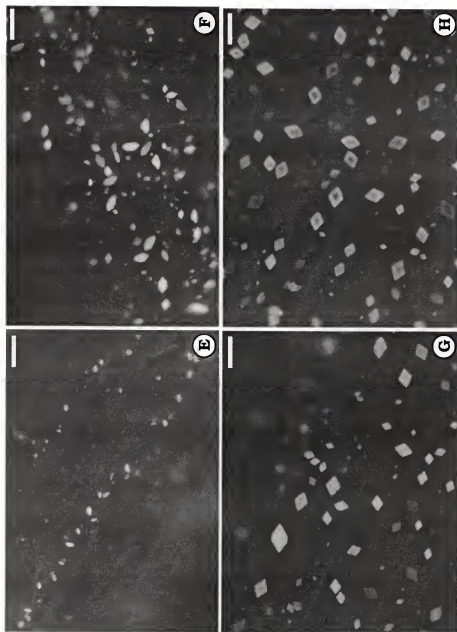


Figure 6-4 continued.

E-F. Primordium length = 3 and 4mm. respectively. Note shape of developing crystals.

G-H. Primordium length = 6 and 9mm. respectively. Note large size of crystals.

E-H. Bars = 10 μ m.

Crystals of this size have not been observed in container-grown plants (Chapter 4), nor are they detected in mineral-deficient plants grown in 3mM Ca^{2+} (Figure 6-3D). Another difference is the significantly larger number of intracellular raphide idioblasts in mineral-deficient plants grown in 7mM Ca^{2+} (Table 6-1 and Figure 6-4C).

The extraplasmic crystal maturation zone in primordia of equal lengths from mineral-deficient plants grown under 7mM Ca^{2+} occurred in physiologically younger cells compared to mineral-deficient plants grown under 3mM Ca^{2+} (Figure 6-3B). Total extraplasmic crystal number per epidermal cell is similar, however, the number of large crystals is significantly higher in mineral-deficient plants grown under 7mM Ca^{2+} (Table 6-1). Young primordia (3-4mm in length) in mineral-deficient 3 and 7mM Ca^{2+} treatments exhibit extraplasmic crystals and raphide idioblasts formation (Table 6-1 and Figures 6-3A, 6-4A). The shape of developing extraplasmic crystals in these primordia is truncated rhombohedral and hexagonal (Figures 6-3C, 6-4E-F). In mature epidermal cells of mineral-deficient plants grown in 3 and 7mM Ca^{2+} the extraplasmic crystal shape was rhombohedral to pentagonal and hexagonal (Figures 6-3D, 6-4G-H).

The non-deficient group of plants does not differ dramatically with respect to extraplasmic crystal and raphide idioblast deposition (Table 6-2). The crystal maturation zone occurs earliest in leaf primordia of plants grown under 7mM Ca^{2+} (Figure 6-7A-B), compared to plants grown in 0 mM Ca^{2+} (Figure 6-5C) and 3mM Ca^{2+} (Figure 6-6A). The most pronounced and significant difference is the number of intracellular raphide idioblasts. Highest values always are detected in plants grown in 7mM Ca^{2+} (Figures 6-5C, 6-6A, 6-7B).

Table 6-2. Cuticular- and raphide-related characteristics of leaf primordia from non-deficient *Dracaena sandariana* plants grown in three exogenous levels of Ca^{2+} .

Treatment	Crystal Maturation Zone (μm from primordium base)	Crystal Shape	Crystal Size	Crystal Number (per epidermal cell)	Number of Raphide Idioblasts
				<1-2 μm 3-5 μm 6-10 μm	$\approx$ 1-4mm ^w $\approx$ 5-14mm
				Total*	
0mM Ca ²⁺	2550 ^z	Rhombohedral ^y to 5- or 6-sided	<1-10 μm	ns 28 ^x ns 18 s 4	s 2 ^v s 10
3mM Ca ²⁺	2090	Rhombohedral to 5- or 6-sided	<1-10 μm	25 21 6 50 ^{ns}	6 25
7mM Ca ²⁺	1970	Rhombohedral to 5- or 6-sided	<1-10 μm	24 23 10 57 ^{ns}	10 32

^z Values are means of three replicates. Measurements were taken on primordia between 10-14mm in length.

^y Rhombohedral (diamond-shaped) with four sides as seen in surface view.

^x Values are means (rounded to integers) of three primordia, five epidermal cells per primordium.

^w Raphide numbers are counted in primordia with the specified lengths.

^v Values are means rounded to an integer.

*Total crystal number per epidermal cell (the sum of all size crystals).

**Arrows indicate values subjected to statistical comparisons among crystal size groups and total crystal numbers per epidermal cell between Ca^{2+} treatments.

^{ns/s} Means not significant/significant at the 0.05 level.

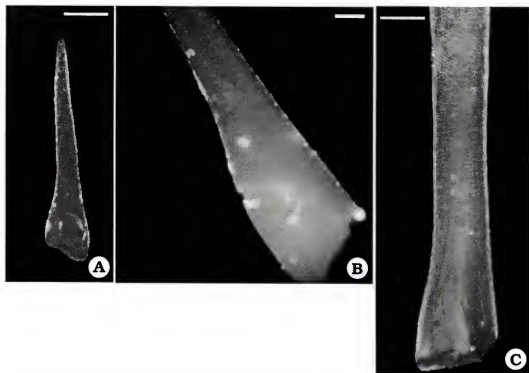


Figure 6-5. LM micrographs of leaf primordia from non-deficient *Dracaena sanderiana* plants grown in 0mM Ca^{2+} .

A-E. Viewed in polarized light with crossed polars.

A-B. Primordium length = 3mm. Note single raphide idioblast. Bars = 500 and 100 μm , respectively.

C. Primordium length = 14mm. Basal portion of the primordium. A few raphide idioblasts are present. Bar = 500 μm .

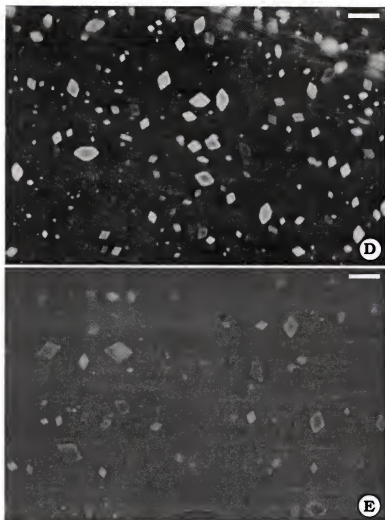


Figure 6-5 continued.

D-E. Primordium length = 14mm.

Note large size of cuticular crystals and concomittant increase in birefringence. Bar = 10 μ m.

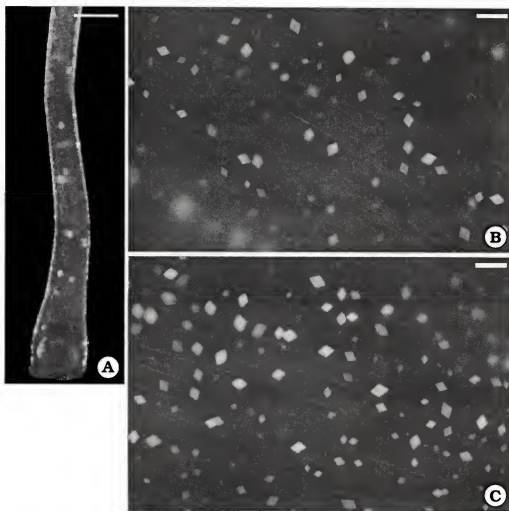


Figure 6-6. LM micrographs of leaf primordia from non-deficient *Dracaena sanderiana* plants grown in 3mM Ca^{2+} .

A-D. Viewed in polarized light with crossed polars.

A. Primordium length = 7mm. Note numerous raphide idioblasts in the basal portion of the primordium. Bar = 500 μm .

B-D. Primordium length = 7mm. Note final size and shape of cuticular crystals. Bar = 10 μm .

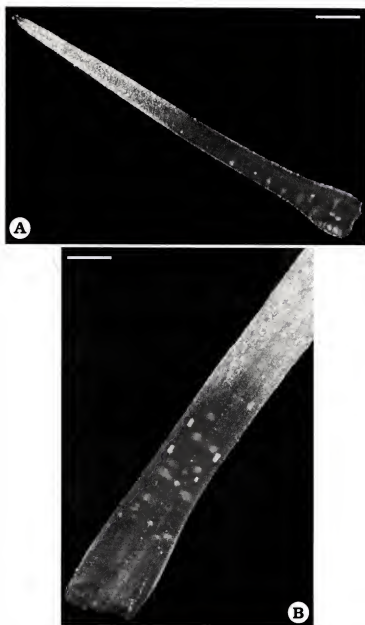


Figure 6-7. LM micrographs of leaf primordia from non-deficient *Dracaena sanderiana* plants grown in 7mM Ca^{2+} .

A-D. Viewed in polarized light with crossed polars.

A. Primordium length = 4mm. Note presence of raphide idioblasts. Bar = 500 μm .

B. Primordium length = 12mm. Basal portion of the primordium. Note presence of raphide idioblasts. Bar = 500 μm .

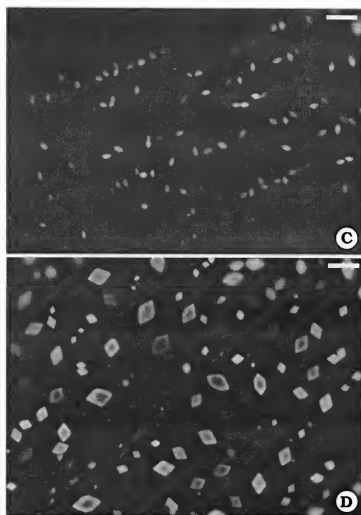


Figure 6-7 continued.

C-D. Primordium length = 12mm. Note shape of developing crystals in C and large size and high birefringence of crystals in D. Bars = 10 μ m.

All non-deficient plants have some extraplasmic crystals with truncated rhombohedral and hexagonal shapes (Figure 6-7C). These shapes and the more common rhombohedral one, are found in fully-formed extraplasmic crystals (Figures 6-5D-E, 6-6B-C, 6-7C-D). Final crystal size reaches 10µm in non-deficient plants from all treatments, although the highest number of large crystals is found in the 7mM Ca^{2+} group (Table 6-2). The increase in crystal size is reflected in the increase in birefringence, which exhibit interference colors of second order (Figure 6-5E). Total number of extraplasmic crystals per epidermal cell is not significantly different among non-deficient plants in the three Ca^{2+} concentrations.

Discussion

The depletion of internal stored Ca^{2+} has a dramatic effect on the deposition of CO in the extraplasmic and the intracellular forms. An 18 month-period is needed to deplete reserve Ca^{2+} as *D. sanderiana* grows very slowly. The plants remain healthy during this time, and the only visible symptom of nutrient stress was a progressive reduction in leaf size. During the Ca^{2+} partitioning KNO_3 is added to promote growth. At termination of the experiment, mineral-deficient plants grown in the three Ca^{2+} levels are very similar in leaf size and leaf number. However, leaves are approximately one-third the size of leaves from the non-deficient group. Also the mineral-deficient plants have a larger root mass than the non-deficient group.

Despite the fact that no Ca^{2+} was present in the growing solution, mineral-deficient plants still formed extraplasmic crystals. However, that process resulted in a reduction of the intracellular raphide bundles. The

extraplasmic crystals were initiated in numbers very similar to that found in mineral-deficient plants grown in media supplemented with Ca^{2+} , but their size is considerably smaller. The rhombohedral shape of developing extraplasmic crystals in mineral-deficient plants is intriguing because it implies that the growth rates of certain crystal faces changes in response to the low, and probably the slow supply of Ca^{2+} ions. Such a drastic response is not observed in the remaining two treatments of mineral-deficient plants, nor in the three treatments of non-deficient plants (Figures 6-2J, 6-3D, 6-4H, 6-5C, 6-6C, 6-7D).

The extraplasmic crystal maturation zone occurs later in the leaf primordial ontogeny in mineral-deficient plants. The significance of this fact is that crystal deposition is delayed and does not proceed in accord with normal development of the epidermal cells. As a result smaller crystal size may be due to the fact that the developing epidermal cells have differentiated to the stage where deposition of cellulose material in the apoplastic space has been initiated, and the extraplasmic crystals become spatially separated from a supply of ions in the living protoplasm, and cannot achieve a larger size. Conversely, the large crystal size ($10\mu\text{m}$) found in mineral-deficient and non-deficient plants grown in 7mM Ca^{2+} , can be caused by the crystal nucleation occurring precociously in leaf development, and ample amounts of growing time and ion supply are available.

The extraplasmic crystal maturation zone appears in young leaf primordia (3-4mm in length) of mineral-deficient and non-deficient plants grown in 3 and 7mM Ca^{2+} (Figures 6-3A; 6-4A; 6-7A). This event occurs earlier compared to crystal deposition in container-grown plants using recommended

horticultural conditions (Chapter 4). This finding implies that the deposition of extraplasmic crystals can be induced at an earlier ontogenetical stage by raising exogenous Ca^{2+} levels.

The total number of extraplasmic crystal per epidermal cell does not differ significantly among all plants from all treatments. This finding implies that nucleation sites are pre-determined and finite in number. Conversely, the number of intracellular raphide idioblasts is highly flexible. Highest raphide idioblast numbers are found in mineral-deficient plants grown in 7mM Ca^{2+} . This observation may reflect the plant's response to Ca^{2+} deprivation, and its strategy to sequester as much Ca^{2+} as possible. This higher rate of Ca^{2+} accumulation also can be caused by a decrease and/or a delay in the operation of the normal Ca^{2+} -utilizing shunts.

In terms of prioritization, the extraplasmic COM crystals took precedence over the intracellular COM raphides. This observation is most obvious in plants which have been depleted of Ca^{2+} . As the supply of Ca^{2+} ions increased, higher numbers of raphide idioblasts develop in both mineral-deficient and non-deficient plants. The non-deficient plants grown in 0 mM Ca^{2+} presumably obtained Ca^{2+} from internal sources, possibly the raphides, as evidenced by the decrease in their number.

Raphide idioblast formation is known to be rapid and reversible in *Lemna minor* roots (Franceschi, 1989). Entire new raphide bundles were formed within 30 minutes of induction in *Lemna minor* roots in medium supplemented with 5 and 7mM Ca^{2+} . This finding contrasts with the 3 to 5 days required after the inductive stimulus (elevated exogenous Ca^{2+} levels) in peeled *Gleditsia triacanthos* and *Albizia julibrissin* leaflets (Borchert, 1985). The dissolution of

raphide crystals in Franceschi's study, however, took considerably longer, and was achieved by exposing *Lemna* roots to Ca-ionophore. Experiments with *Canavalia ensiformis* showed that the number of crystal idioblasts could decrease in half when plants were grown in low Ca^{2+} supply (0.2 meq/Ca/l) (Frank, 1972). Franceschi and Horner (1979) reported that Ca^{2+} could induce raphide idioblast formation in *Psychotria punctata* callus.

This study represents the first attempt to determine the effects of exogenous Ca^{2+} supply in *D. sanderiana*, a species that features two or more CO types in several locations of the plant body. This study showed that mineral-deprived plants favor extraplasmic COM over intracellular raphide COM formation. This finding supports the previously-promoted hypothesis that crystal-forming cells provide a mechanism for regulation of Ca^{2+} levels in plant tissues (Franceschi, 1989), and act as a storage depot for Ca^{2+} ions.

Future studies should focus on the intracellular COD crystals and their role in Ca^{2+} partitioning in *D. sanderiana*. It would be valuable to determine the precise allocation and transport patterns of Ca^{2+} and oxalate ions in the apical meristem and the youngest leaf primordia using radioactively-labeled compounds in a tissue culture system.

CHAPTER 7

CALCIUM OXALATE CUTICULAR DEPOSITS IN THE GENUS *DRACAENA*

Results

Dracaena species used in this study were obtained from two general sources. The first group includes species commonly grown and cultivated in the United States, and the second group included species from Spain and Gran Canaria, the so-called 'Tree Dracaenas'. Although *D. arborea* and *D. draco* belong to the 'Tree Dracaenas', they were placed in the first group because of their widespread cultivation in the USA. Leaf samples were obtained from a local nursery (*D. arborea*), and from the UF greenhouse conservatory (*D. draco*). Epidermal cell characteristics and apoplastic crystal characteristics for the fourteen *Dracaena* species examined are listed in Table 7-1.

The cuticular deposits in the first group (listed in alphabetical order) vary with respect to quantities and size among *Dracaena* species (Figure 7-1). All examined species possess cuticular crystals. 'Tree Dracaenas' exhibit almost identical characteristics among themselves with respect to epidermal cell dimensions and crystal deposition (Figure 7-1A-B,K-N). Their leaf morphology is also very similar among themselves.

Among the cultivated species, the largest cuticular crystals belong to *D. thalioides* (Figure 7-1J). Similarly, *D. marginata*, *D. reflexa* and *D. sanderiana*, exhibit fewer but larger cuticular deposits (Figure 7-1E,G-H) when compared to *D. arborea*, *D. deremensis*, *D. fragrans*, and *D. surculosa* (Figure 7-1A-B,D,I).

Table 7-1. Comparative analysis of cuticular crystal and epidermal cell characteristics in fourteen *Dracaena* species.

Species Name	Epidermal Cell Shape and Length to Width Ratio	Crystal Quantity (per 100µm ² of epidermal cell area)	Crystal Size	Crystal Birefringence and Morphology	Crystal Orientation (long crystal axis with respect to the long cell axis)
<i>D. arborea</i> Willd. (Figure 7-1A)	elongated 5:1 - 7:1	30 - 40	< 1 - 1µm	high	random
<i>D. deremensis</i> Engl. (Figure 7-1B)	elongated 2.7:1 - 4.5:1	20 - 30	< 1 - 2µm	high	random
<i>D. draco</i> L. (Figure 7-1C)	elongated 6:1 - 8:1	50 - 100	< 1 - 1µm	high	random
<i>D. fragrans</i> (L.) Ker-Gawl. (Figure 7-1D)	rounded 1.2:1 - 2:1	10 - 20	< 1 - 3µm	high; consistent with COM	random
<i>D. marginata</i> Lam. (Figure 7-1E)	elongated 10:1 - 15:1	5 - 15	< 1 - 5µm	very high; consistent with COM	less random; some of the largest crystals to cell axis and equidistant from longitudinal cell walls
<i>D. massifiana</i> (Figure 7-1F)	elongated and rounded 1.6:1 - 6:1	10 - 25	< 1 - 2µm	high	random
<i>D. reflexa</i> Lam. (Figure 7-1G)	elongated 5:1 - 7:1	5 - 20	< 1 - 7µm	high	less random; some of the largest crystals to cell axis and equidistant from longitudinal cell walls

Table 7-1 continued.

Species Name	Epidermal Cell Shape and Length to Width Ratio	Crystal Quantity (per 100µm ² of epidermal cell area)	Crystal Size	Crystal Birefringence and Morphology	Crystal Orientation (long crystal axis with respect to the cell walls)
<i>D. sanderiana</i> hort Sander ex M.T. Mast. (Figure 7-1H)	elongated 10:1 to 15:1	2 - 5	< 1 - 6µm	very high; consistent with COM	less random; most larger crystals to cell axis and equidistant from longitudinal cell walls
<i>D. surculosa</i> Lindl. (Figure 7-1I)	elongated 2.7:1 - 5:1	2 - 7	< 1 - 2µm	high	random
<i>D. thalioides</i> Hort. Makoy ex E. Morr. Figure 7-1J)	elongated 5:1 - 7:1	2 - 10	< 3 - 12µm	very high; consistent with COM	less random; some of the largest crystals to cell axis and equidistant from longitudinal cell walls
<i>D. cinnabari</i> Balf. (Figure 7-1K)	elongated 6:1 - 8:1	50 - 100	< 1 - 1µm	high	random
<i>D. ellembekkiana</i> Hort. Ex Figure 7-1L)	elongated 6:1 - 8:1	50 - 100	< 1 - 1µm	high	random
<i>D. ombet</i> Kots. & Peyr. (Figure 7-1M)	elongated 6:1 - 8:1	50 - 70	< 1 - 1µm	high	random
<i>D. tamaranae</i> Marrero, Almeida & Glez.-Martin. (Figure 7-1N)	elongated 6:1 - 8:1	50 - 100	< 1 - 1µm	high	random

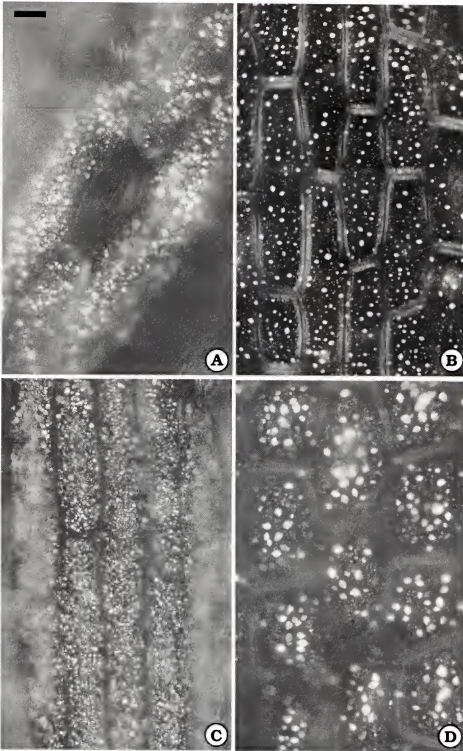


Figure 7-1. LM micrographs of epidermal peels of *Dracaena* species (in alphabetical order). Viewed in polarized light with crossed polars. Bar = 10 μ m.

A. *D. arborea* Willd. (L.)

B. *D. deremensis* Engl.

C. *D. draco* L.

D. *D. fragrans* (L.) Ker-Gawl.

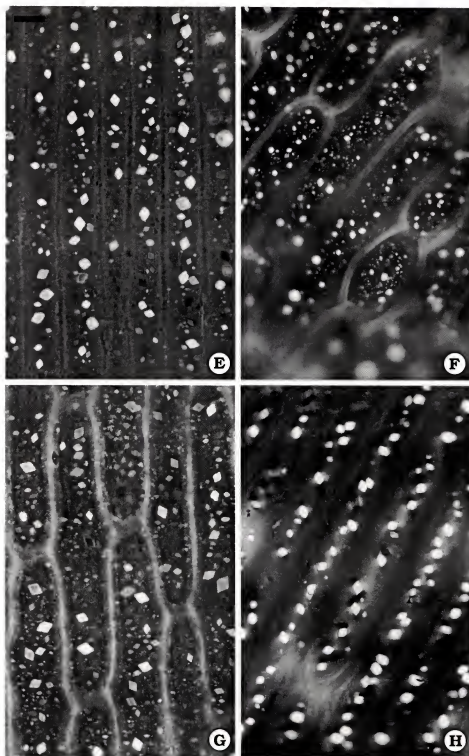


Figure 7-1 continued.

E. *D. marginata* Lam. Bar = 10 μ m.

F. *D. massefiana* (*D. fragrans* cv. 'Massangeana' x *D. surculosa*)

G. *D. reflexa* Lam.

H. *D. sanderiana* Hort. Sander Ex M.T. Mast.

Curiously, *D. massefiana* which is a hybrid between *D. fragrans* and *D. surculosa*, displays features of both species with respect to crystal deposition and epidermal cell characteristics (Figure 7-1F). The fewest crystals per unit cell area are evident in *D. sanderiana* and *D. surculosa* (Figure 7-1H-I). Crystal quantity is highly variable even in the same species depending on where the counts are taken. For example, *D. sanderiana* has large crystals located predominantly along the midline of the epidermal cells. Crystals in *D. marginata* and *D. thalioides* are distributed more uniformly with respect to the epidermal cell area. However, in most species, the largest deposits are located in areas distal from the longitudinal cell walls.

In all species cuticular crystals exhibit high birefringence especially where crystal size is large (e.g., *D. thalioides*). Along with crystal morphology, these two facts indicate that these deposits are likely COM. Their location beneath the cuticle is identical in all examined species and is probably is similar to the crystal location described in *D. sanderiana* (Chapter 4).

Species from the 'Tree group' have epidermal cells with length to width ratios of 5:1 to 8:1. Epidermal cells of *D. fragrans* have the smallest length to width ratio. The orientation of crystal deposits is random in species, which display numerous minute apoplastic crystals. Orientation of the deposits is less random in species, which possess larger but fewer crystals. Excluding *D. fragrans*, the other species studied possess epidermal cells with length to width ratios of approximately 3:1 to 15:1. *D. fragrans* is an exception to these general trends as it has large but randomly distributed crystals.

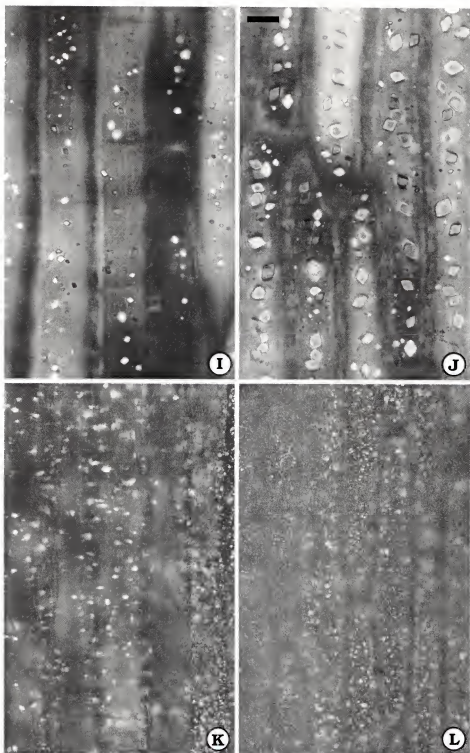


Figure 7-1 continued.

I. *D. surculosa* Lindl.

J. *D. thalioides* Hort. Makoy ex E. Morr. Bar = 10 μ m.

K. *D. cinnabari* Balf.

L. *D. ellembeckiana* Hort. ex

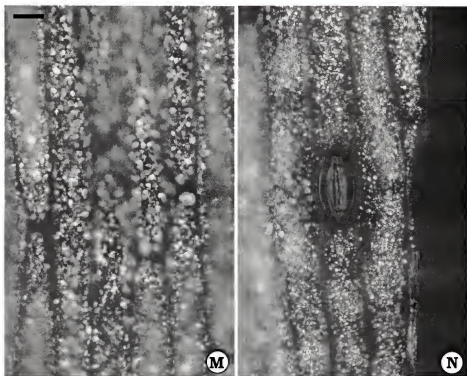


Figure 7-1 continued.

M. *D. ombet* Kots. & Peyr. Bar = 10 μ m.

N. *D. tamaranae* Marrero, Almeida & Glez.-Martin.

Crystal orientation is related to epidermal cell shape, (i.e., the greater the length to width ratio and the larger the deposits, the more regular the orientation of the crystals). This observation is readily demonstrated in *D. fragrans* and *D. sanderiana*. The former species has rounded epidermal cells and the cuticular crystals are randomly distributed (Figure 7-1D). In contrast, the latter species has elongated epidermal cells and the crystals (which are of comparable in size) are lined up and equidistant from the longitudinal cell walls (Figure 7-1H).

No relationship between epidermal cell size and amount of crystals is evident. However, species of the 'Tree Dracaenas' deposit the largest quantity of uniformly small cuticular crystals. The distinction between individual species within this group, based solely on crystal numbers and size, is not reliable. Crystal deposits in other species of *Dracaena* had species-specific characteristics. This fact, in combination with leaf epidermal characteristics, could be taxonomically important in the genus *Dracaena*.

Discussion

Detailed studies investigating the taxonomic value of CO deposits in a given genus or a family are infrequent. Genua and Hillson (1985) surveyed 14 species in Araceae family and recorded the presence of druses, raphides, prismatics, and crystal sand in all species. They also reported results from chemical analysis of the crystal deposits using two staining methods. Hentzelman and Howard (1948) described CO crystal occurrences in species of Icacinaeae. Chattaway (1955, 1956) gave detailed and illustration-replete accounts of crystals in representative species of approximately 1000 woody

genera of 160 families. She pointed out that some crystal types, such as druses and raphides, and certain crystal arrangements, (e.g., large crystals accompanied by smaller ones), may not be common. Chattaway concluded that all those characteristics were valuable features in an identification key of the taxa.

Recently, Ilarslan and Horner (1999) reported that in representative species of Rosaceae grown in Turkey the intracellular CO crystals showed species-specific locations, shapes, and numbers within the leaf lamina. They concluded that the patterns of crystal deposition and crystal characteristics "may be of major significance in sorting out the taxonomy and phylogeny" in the family Rosaceae.

Few studies have examined apoplastic crystal deposits in angiosperm species of the same genus or a family. Ihlenfeldt and Hartmann (1980) found crystalline incrustations in the outer epidermal wall of several species of Mesembryanthemaceae (see Chapter 2, Figure 2-6). The distribution of CO crystals in the cellulose layer in this family is somewhat different than the corresponding distribution in *Dracaena* species, where most crystals are external to the outer cell wall. However, the presence of some CO crystals in the epidermal cell walls in *Dracaena* species cannot be excluded. Additionally, Mesembryanthemaceae species possess thicker outer cell walls, and that may be a contributing factor for the location of the deposits. The large quantities of CO crystals illustrated in their drawings is similar to the crystal deposition in the 'Tree Dracaenas' group. The presence of crystals in the region between the epidermal cell wall and the cuticle is considered a typical adaptational feature for xerophytic habitats (see Chapter 2, Figure 2-5). *Dracaena* species exhibit

adaptations consistent for xerophytic habitat in structural characteristics of the cuticular layer (see Chapter 4) (Ihlendfelt and Hartmann, 1980).

Berg (1994) included a short survey of 8 species from three genera of Casaurinaceae. Although some differences in crystal distribution existed among certain plants, he found that CO crystals embedded in the outer epidermal cell wall in all species. In two genera the crystal distribution was distinct enough to suggest taxonomic importance for "at least some species".

The present study embraced 14 species of *Dracaena*. Detection of cuticular crystals in all species studied indicates that this condition is almost certainly ubiquitous in the genus. Future research efforts should include different *Dracaena* species, with emphasis on crystal patterns and quantification of the cuticular apoplastic crystals.

CHAPTER 8 SUMMARY AND CONCLUSIONS

Formation of CO crystals in leaves of *Dracaena sanderiana* was found to be highly specific and predictable with respect to the location of the various crystal types and relative timing of their development during leaf ontogeny. The present study is the first attempt to isolate and characterize cuticular crystals in a plant species. The hydration state of these crystals is calcium oxalate monohydrate. Their morphology does not differ from COM crystals grown synthetically. The expression of the typical faces $\{101\}$, $\{010\}$, $\{110\}$, and $\{011\}$ was observed in cuticular crystals in *D. sanderiana*.

The observed polarity of crystals with respect to their cuticular orientation strongly suggests biologically-controlled crystal precipitation. The existence of crystal envelopes supports this interpretation. The plane of *b* and *c* axes in cuticular crystals is parallel to the long axis of the epidermal cell/leaf, while the *a* axis is perpendicular. Such preferential orientation of the crystallographic axes implies biological control over the biomineralization process.

The predominant features of immature crystal-excreting epidermal cells of *D. sanderiana* are paramural bodies with variously electron-dense contents, an abundance of RER, large quantities of cytoplasmic ribosomes, and clusters of dictyosomes with many associated vesicles. The overall array of subcellular

structures is highly suggestive of a very dynamic system involved in crystal precipitation.

Initial events in the deposition of cuticular COM crystals include fusion of numerous pleiomorphic and lamellate vesicular bodies to form rectangularly-shaped electron-dense crystal chambers. The crystal origin is *extraplasmic* since the epidermal cell wall is external to the COM crystals. This study suggests that in *D. sanderiana* cuticular crystallization of COM occurs in crystal chambers, which originate from the plasma membrane.

Growth of cuticular crystals in *D. sanderiana* involves RER-derived vesicles. These vesicles transit the plasma membrane on their way to the apoplastic space and the developing crystals. The involvement of RER-derived vesicles in the crystal deposition process implies active participation of the living protoplast.

Intracellular COD crystals in *D. sanderiana* have a morphology typical of the tetragonal-dipyramidal class with expression of $\{101\}$ faces enclosing two tetragonal pyramids at both crystal ends. Development of some unexpected faces also is noted, namely, $\{100\}$ faces enclosing the tetragonal prism. The unusual crystal morphologies in the COD crystals in *D. sanderiana* result from expression of $\{001\}$, $\{111\}$, and $\{110\}$ faces that has not been documented previously. Precipitation of COD inside *D. sanderiana* cells is highly controlled as evidenced by the elaboration of RER crystal vacuoles and the fact that crystal morphology is modified by the development of unstable crystal faces.

Raphide-containing cells in *D. sanderiana* exhibited characteristics typical of System II crystal idioblasts, namely, lamellate sheaths around the

chamber walls, mucilage-like material surrounding the developing crystal chambers, and paracrystalline bodies with closely spaced subunits.

Dracaena sanderiana plants responded to exogenous Ca^{2+} levels in remarkably different ways depending on whether or not they had been deprived of endogenous Ca^{2+} . Calcium-deficient plants deposited cuticular crystals at the expense of the intracellular raphide bundles. The number of extraplasmic crystals in calcium-deficient plants grown in Ca^{2+} -supplemented nutrient solutions versus non-supplemented were similar, but crystals were considerably smaller in plants grown in the non-supplemented solutions. The rhombohedral shape of growing extraplasmic crystals in calcium-deficient plants grown in 0 mM Ca^{2+} implies that the growth rates of certain crystal faces is modulated by ionic Ca^{2+} levels.

The extraplasmic crystal maturation zone occurred later in the leaf primordial ontogeny in calcium-deficient plants grown in 0 mM Ca^{2+} than in calcium-nondeficient plants grown in 3 or 7mM Ca^{2+} . Thus crystal deposition is not rigidly associated with the physiological age of differentiating epidermal cells, but with the Ca^{2+} ions supply. The smaller crystal size in calcium-deficient plants may be a result of spatial separation from the living protoplasm. Conversely, the large crystal size (10 μm) found in calcium-deficient and non-deficient plants grown in 7mM Ca^{2+} , could be related to precocious crystal nucleation induced by elevated ionic Ca^{2+} concentrations.

The extraplasmic crystal maturation zone appears in young leaf primordia (3-4mm in length) of mineral-deficient and non-deficient plants grown in 3mM and 7mM Ca^{2+} . This event occurs earlier compared to crystal deposition in container-grown plants using recommended fertilization

practices. This finding provides additional support for the premise that the deposition of extraplasmic crystals is modulated by ionic Ca^{2+} levels and can be induced at an earlier ontogenetical stage by raising exogenous Ca^{2+} levels or delayed by lowering exogenous Ca^{2+} levels.

The total number of extraplasmic crystals per epidermal cell did not differ significantly between treatments. This implies that nucleation sites are pre-determined and finite in number. In contrast, the number of intracellular raphide idioblasts was highly variable. In terms of prioritization, the cuticular COM crystals take precedence over the intracellular COM raphides, and was most obvious in plants that had been calcium-deprived.

Detection of cuticular crystals in 14 examined species of *Dracaena* indicates that it is almost certainly ubiquitous in the genus and species of the 'Tree Dracaenas' deposit the largest quantity of uniformly small cuticular crystals. The distinction between individual species within this group, based solely on crystal numbers and size, however, is not reliable. All other species of *Dracaena* studied did display species-specific quantities and size of cuticular crystals. This fact, in combination with leaf epidermal characteristics, could be taxonomically important in the genus *Dracaena*.

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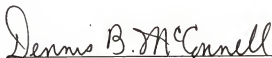
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BIOGRAPHICAL SKETCH

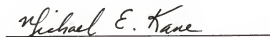
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Dennis B. McConnell, Chair
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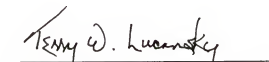
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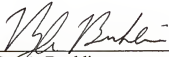
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December 1999



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